An assessment of ischemia-reperfusion injury in rats exposed to chronic psychological stress

Lukas Van Zyl Olivier

Thesis presented in partial fulfillment of the requirements for the degree of Master of Science (Physiology) in the Faculty of Natural Science at Stellenbosch University.

Supervisor Prof M. Faadiel Essop

December 2019

Table of contents

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature: Date: December 2019

Acknowledgments

Prof. Faadiel Essop (supervisor): Thank you for the opportunity you gave me to complete this masters. These 2 years have taught me more than I could have ever imagined. Thank you for always having an open door and listening to all our problems.

Mr. Lucien Sher: Thank you for making this masters as enjoyable as it was. You made lab work that much more bearable. Thank you for all the laughs that got us through the tough times (there were many). I would not have been able to do this without you.

Dr. Danzil Joseph: Thank you for all the help with our lab work and always being willing to help out. Thank you for all your advice and support throughout these two years.

CMRG: Thank you to everyone in CMRG for your input, ideas, critiques and friendships.

My parents: Thank you for my education. Not just the two years of masters, but my entire educational journey. Without your help I would never have succeeded. Thank you for all the support you have given me these past two years.

Oxidative stress unit (CPUT): Thank you to Mr. Fanie Rautenbach for allowing us to complete all our oxidative stress analyses in your lab. Thank you for answering all our questions and explaining everything so well.

Dr. Erna Marais: Thank you so much for all the help with the perfusions and staining. You have taught me so much in so little time. Thank you for all the guidance and suggestions.

Mr. Noel Markgraaff: Thank you for allowing us to house our animals in your facility and for always being willing to help us. Your advice and assistance made this study the success it is.

List of abbreviations

Ach	Acetyl choline
ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AR	Area at risk
ATP	Adenosine triphosphate
BPM	Beats per minute
C5	Complement fragment 5
CD4	Cluster of differentiation 4
CRF	Corticotropin-releasing factor
CRP	C-reactive protein
DAMP	Damage associated molecular patterns
DNA	Deoxyribonucleic acid
E	Epinephrine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
H_2O_2	Hydrogen peroxide
HPA	Hypothalamic-pituitary-adrenal
I	Infarct zone
ICAM	Intracellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase

IL	Interleukin
LAD	Left anterior descending
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MR	Mineralocorticoid receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOX	NADPH oxidase
NSTEMI	Non-ST-elevation myocardial infarction
O_2^-	Superoxide anion
$ONOO^-$	Peroxynitrite anion
PGE_2	Prostaglandin E2
PNS	Parasympathetic nervous system
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
ROS	Reactive oxygen species
SEM	Standard error of the mean
SNS	Sympathetic nervous system
STEMI	ST-elevation myocardial infarction
TBARS	Thiobarbituric acid reactive substances
TLR	Toll-like receptor
TNFA	Tumor necrosis factor alpha
UCMS	Unpredictable chronic mild stress
V	Viable tissue

VCAM	Vascular cell adhesion molecule
WHO	World health organization
XO	Xanthine oxidase

List of figures

Figure 3.2	**Food consumption over the eight week protocol (Sher and Olivier, unpublished data).** Data presented as mean ± SEM; statistical analysis: repeated measures, two-way ANOVA, Bonferroni post hoc; *p<0.05, ***p<0.001; n = 12.
Figure 3.3	**Percentage recovery of functional ability after ischemia and reperfusion.** Data displayed as mean ± SEM; statistical analysis: unpaired t-test; n = 10-11.
Figure 3.4	**Size of infarct (A) and area at risk (B) displayed as a percentage**. Data displayed as mean ± SEM; statistical analyses: unpaired t-test, Bonferroni post hoc; *p<0.05; n = 12.
Figure 3.5	**Plasma corticosterone results of this study (A) (n = 6) and plasma corticosterone of joint study (Sher and Olivier, unpublished data) (B) (n = 12).** Data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 12.
Figure 3.6	**Plasma ACTH results of this study (A) (n = 6) and plasma ACTH of joint study (Sher and Olivier, unpublished data) (B) (n =12).** Data presented as mean ± SEM; statistical analyses: unpaired t-test, Bonferroni post hoc; *p<0.05. ACTH: adrenocorticotropic hormone.
Figure 3.7	**Oxidative stress analyses. Malondialdehyde concentration (A) and superoxide dismutase activity (B) in spleen tissue**. Data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 12.
Figure 3.8	**Plasma concentration of hs-CRP** . Data displayed as mean ± SEM; statistical analysis: unpaired t-test; n = 12. CRP: C-reactive protein.

List of tables

Foreword

The following thesis will be divided into two main sections. Chapter 1 will serve as a review article while Chapter 2 will contain all our acquired results and serve as a separate article.

Chapter 1

Inflammation and oxidative stress as links between chronic stress and aggravated ischemia-reperfusion injury.

ABSTRACT

Cardiovascular disease remains the leading cause of death worldwide. Apart from known risk factors such as poor dietary intake, physical inactivity and smoking, chronic psychological stress is emerging as an important modifiable risk factor in the development of cardiovascular disease. The body relies on two physiological mechanisms to counter acute stressors and to achieve and/or maintain homeostasis, i.e. the hypothalamic-pituitary-adrenal axis and the autonomic nervous system. However, chronic activation of these systems can lead to the disruption of cellular and systemic processes that could potentially result in the development of neurological or psychosomatic diseases. Both chronic stress and ischemia-reperfusion injury are associated with a robust inflammatory response and the induction of oxidative stress. Does chronic psychological stress render the heart more susceptible to ischemia and reperfusion damage, and what are the role(s) of oxidative stress and inflammation in stress-related cardiac dysfunction? These questions will form the basis of this review. Following a comprehensive review, we established that chronic stress does render the heart more susceptible to damage following ischemia-reperfusion. After reviewing the mechanisms involved in both ischemia-reperfusion and chronic stress, we hypothesized that chronic stress induced inflammation and oxidative stress are major contributors in aggravated ischemia-reperfusion injury.

OPSOMMING

Kardiovaskulêre siektes is jaarliks die grootste oorsaak van dood wêreldwyd. Behalwe die bekende risikofaktore soos swak dieetinname, fisiese onaktiwiteit en rook, verskyn kroniese psigologiese stres as 'n belangrike veranderlike risikofaktor in die ontwikkeling van miokardiale infarksie. Die liggaam maak staat op twee fisiologiese meganismes om akute stressors teen te werk en homeostase te bewerkstellig of te handhaaf, naamlik die hipotalamus-pituïtêre-byniere-as en die outonome senuweestelsel. Alhoewel dit 'n gekoördineerde fisiologiese reaksie is wat homeostase handhaaf, kan kroniese aktivering van hierdie stelsel lei tot die ontwrigting van sellulêre en sistemiese prosesse wat moontlik kan lei tot die ontwikkeling van neurologiese of psigosomatiese siektes. Beide kroniese sielkundige stres en iskemie-reperfusiebesering word geassosieer met 'n sterk inflammatoriese reaksie en die induksie van oksidatiewe stres. Maak kroniese psigologiese stres die hart meer vatbaar vir skade na iskemie en reperfusie, en wat is die rol van oksidatiewe stres en inflammasie in stresverwante disfunksie van die hart? Hierdie vraag vorm die basis van hierdie oorsig. Na 'n omvattende oorsig het ons vasgestel dat kroniese stres die hart meer vatbaar maak vir skade na die induksie van iskemie-herperfusie. Na die hersiening van die meganismes wat betrokke is by iskemie-reperfusie en kroniese stres, het ons die hipotese vasgestel dat kroniese stres-geïnduseerde inflammasie en oksidatiewe stres belangrike bydraers is tot die verergering iskemie-reperfusie skade.

1. Introduction

Cardiovascular disease remains the leading cause of death worldwide (WHO, 2016). Apart from known risk factors such as poor dietary intake, physical inactivity and smoking, chronic psychological stress is emerging as an important modifiable risk factor in the development of cardiovascular disease (Yusuf *et al.*, 2004). Here the focus is on acute coronary syndrome that describes any condition associated with sudden reduced blood flow to the heart resulting in myocardial infarction (Sanchis-Gomar *et al.*, 2016). The reduced blood flow itself is not the only mediator of damage as the restoration of blood flow (reperfusion) can also elicit detrimental effects (Yellon and Hausenloy, 2007). Although there are several mechanisms implicated in this process the induction of oxidative stress and inflammation were identified as key mediators (Eltzschig and Eckle, 2011; Kalogeris *et al.*, 2014; Granger and Kvietys, 2015). As chronic psychological stress is known to also induce oxidative stress and inflammation in the heart (Schiavone *et al.*, 2013; Salim, 2014; Y. Z. Liu, Wang and Jiang, 2017; Maydych, 2019), two key questions arise: a) does chronic psychological stress render the heart more susceptible to ischemia and reperfusion damage, and b) what are the role(s) of oxidative stress and inflammation in stress-related cardiac dysfunction? These questions will form the basis of this review.

2. Stress

Hans Selye who is known as the modern-day ‘’father of stress’’ defined this in 1974 as the body’s non-specific response to any demand (Goldstein and Kopin, 2007) that can either be of an intrinsic or extrinsic nature (Nicolaides *et al.*, 2015). Homeostasis is subsequently re-established by both physiological (nervous, endocrine and immune systems) and behavioral adaptive responses (Chrousos, 2009; Yang *et al.*, 2015). Selye proposed three general stages of coping with a stressor i.e. the initial “alarm reaction” or “fight-or-flight” response, a stage of adaptation that is associated with habituation of the stressor, and finally a stage of exhaustion or death (Goldstein and Kopin, 2007). This definition

remained relatively constant with the central concept being that the body is able to react to an external and internal stressor(s) by aiming to cope with the additional demands.

Psychological stress is a major global health problem that requires a suitable response to help attenuate its overall impact on health and well-being. Here especially developing nations are hardest hit as shown by a survey (published in 2013) that listed the most stressed countries based on seven equally weighted parameters: homicide rates, income inequality, corruption perception, unemployment, urban air pollution, life expectancy and purchasing-power-parity basis (Bloomberg, 2013). Of note, the survey showed that South Africa was named the second most stressed country in the world, just behind Nigeria (Figure 1.1).

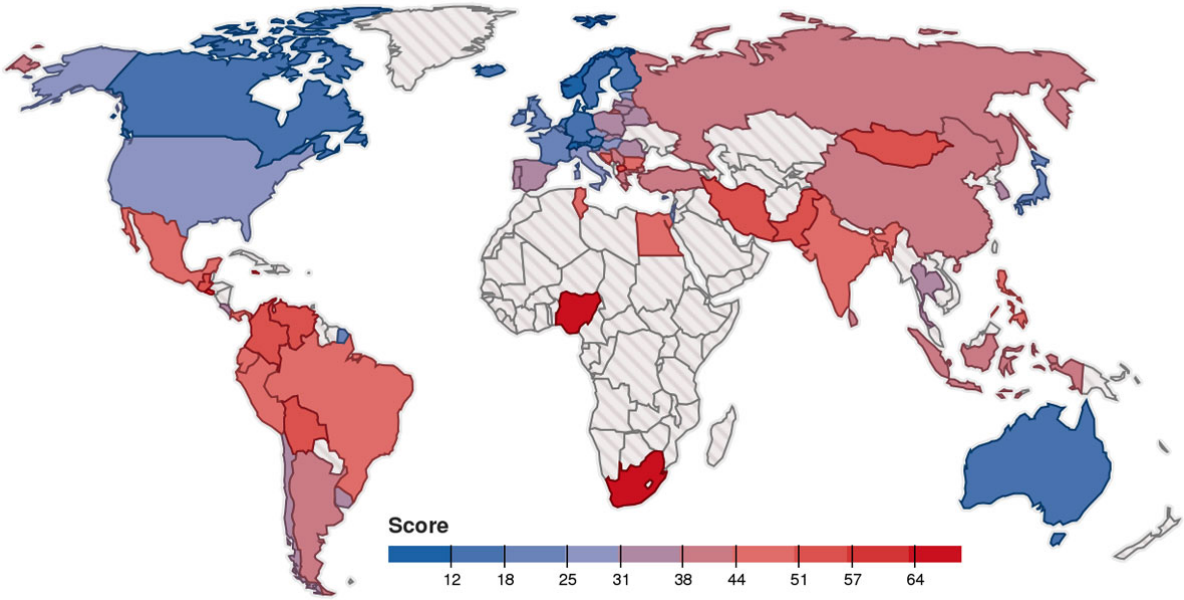


Figure 1.1: The most stressed-out countries – a global perspective (Abdullah., 2016)**.** Scores are based on above mentioned parameters: countries with the least stressful measure for each parameter receive 0 points, with a maximum of 100 points being available for countries with higher stress levels. Adapted from "Bloomberg database" (Bloomberg, 2013).

Keeping this alarming context in mind, it is important to consider the physiological responses mounted in order to attempt to restore homeostasis. The human body relies on two physiological mechanisms to counter acute stressors and to achieve or maintain homeostasis (Chrousos and Gold, 1992; Golbidi, Frisbee and Laher, 2015), i.e. the hypothalamic-pituitary-adrenal (HPA)-axis and the autonomic nervous system (Nicolaides *et al.*, 2015; Agorastos *et al.*, 2018).

2.1. The HPA-axis

The first mechanism operating during a stressful situation is the activation of the HPA-axis (Tsigos and Chrousos, 2002; Agorastos *et al.*, 2018) that involves release of corticotropin releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus. CRF stimulates the production of proopiomelancortin, the precursor for adrenocorticotropic hormone (ACTH). Arginine vasopressin (AVP) works in synergy with CRF in stimulating ACTH secretion from the anterior pituitary (Juruena, 2014; Gaffey *et al.*, 2016). ACTH stimulates the release of glucocorticoids from the adrenal cortex, with cortisol the main glucocorticoid (in humans) that is produced in the zona fasciculata (Juruena, 2014; Nicolaides *et al.*, 2015).

Cortisol elicits numerous downstream effects as part of the adaptive response triggered in response to stress. For example, it induces gluconeogenesis and counteracts insulin to thereby increase blood systemic glucose levels as part of the adaptive response to a stressful scenario(s) (Sherwood, 2010). Under basal conditions, cortisol acts as an anti-inflammatory agent (Straub and Cutolo, 2016). Cortisol mediates its negative feedback actions through association with two corticosteroid receptor subtypes: the glucocorticoid receptor (GR) that is located throughout the brain stem, amygdala, hippocampus and hypothalamus, and the mineralocorticoid receptor (MR) that is mainly located in the hippocampus (Juruena, 2014). Under normal conditions cortisol's negative feedback is largely mediated by the MR while under stressed conditions it is facilitated by the less sensitive GR (Stephens *et al.*, 2012; Juruena, 2014) (Figure 1.2).

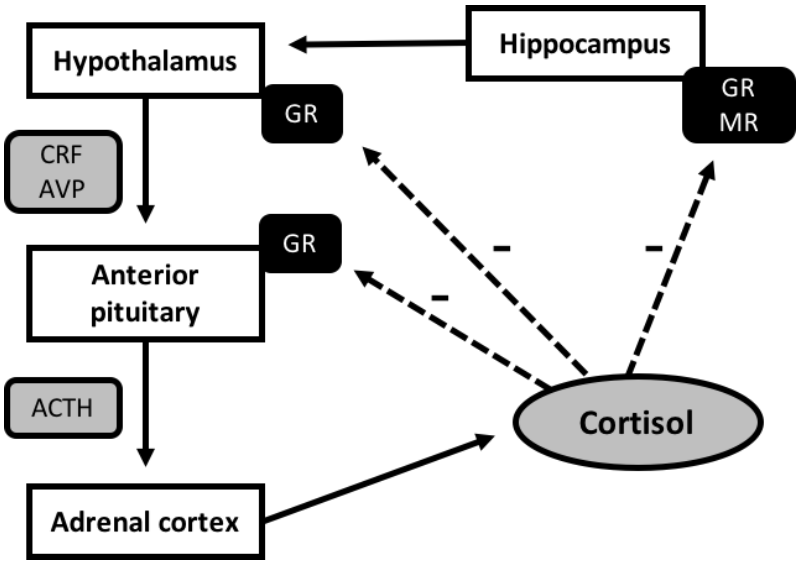


Figure 1.2: The negative feedback mechanisms of cortisol. ACTH: adrenocorticotropic hormone, AVP: arginine vasopressin, CRF: corticotropin releasing factor, GR: glucocorticoid receptor, MR: mineralocorticoid receptor.

2.2. The autonomic nervous system

The second important component operating during the stress response is the autonomic nervous system. Under conditions of stress, sympathetic nervous system activity can increase while there is a corresponding decrease in parasympathetic nervous system activity (Ziegler, 2012; Agorastos *et al.*, 2018). As discussed before, the hypothalamus secretes CRF and CRF-containing neurons then project into the locus coeruleus (Won and Kim, 2016). This in turn increases sympathetic nervous system activity through activation of α1-adrenergic receptors on preganglionic sympathetic neurons and attenuates parasympathetic nervous system activity through the activation of α2-adrenergic receptors on preganglionic parasympathetic neurons (Unnerstall, Kopajtic and Kuhar, 1984; Lewis and Coote, 1990). The main neurotransmitters associated with the autonomic nervous system are norepinephrine, epinephrine and acetylcholine (Won and Kim, 2016). Norepinephrine and epinephrine are released

upon stimulation of the sympathetic nervous system, while acetylcholine is the main neurotransmitter of the parasympathetic nervous system (Won and Kim, 2016). Both epinephrine and norepinephrine increase heart rate and the force of contraction. Epinephrine also dilates blood vessels in skeletal muscle to thereby decrease resistance to blood flow while norepinephrine increases blood flow to skeletal muscle. Both ultimately increase systemic blood pressure (Sherwood, 2010).

Although the HPA-axis and autonomic nervous system function to physiologically adapt the body to cope with the acute internal or external stressors (Schneiderman, Ironson and Siegel, 2005), chronic activation of such mechanisms overrides homeostasis thereby leading to detrimental consequences.

3. Stress-related morbidities

Even though such an adaptive response is a coordinated physiological one that maintains homeostasis, chronic activation of this system can lead to the disruption of cellular and systemic processes resulting in dysfunction of both the nervous system and peripheral organ systems (Duric *et al.*, 2016; Agorastos *et al.*, 2018). Thus chronic stress can potentially lead to dysfunction of the systems that regulate the stress response, ultimately resulting in the development of neurological and psychosomatic diseases (Duric *et al.*, 2016). It can also contribute to the onset of cardiovascular disease, the focus of this review.

Major depressive disorder is increasingly attracting attention as one of the most common causes of disability and affecting close to 16% of the global population (Yang *et al.*, 2015). As a result there is an increased emphasis to delineate underlying mechanisms driving this pathological condition. It is well known that chronic stress evokes neurological changes that are similar to those observed for major depression (Hill *et al.*, 2012). As a result, numerous chronic stress models are being used to investigate the underlying mechanisms contributing to the onset of depression with the aim to develop improved therapeutic interventions (Hill *et al.*, 2012).

The World Health Organization (WHO) recently reported a staggering 17.9 million deaths annually worldwide resulting from cardiovascular disease, constituting 31% of total global mortality (WHO, 2016). There are many factors that contribute to cardiovascular disease development. The most well known risk factors include physical inactivity, unhealthy diet (e.g. high sodium content) and smoking (WHO, 2016). These risk factors may ultimately manifest as high blood pressure, increased blood glucose levels and obesity. Apart from such listed lifestyle-related risk factors, the INTERHEART study also listed chronic psychological stress as a modifiable risk factor for myocardial infarction (Yusuf *et al.*, 2004).

Acute coronary syndrome is a group of clinical symptoms that generally include ST-elevation myocardial ischemia (STEMI), non ST-elevation myocardial ischemia (NSTEMI), and unstable angina (Kumar and Cannon, 2009). A systematic review found that acute mental stress increased platelet activation and endothelial dysfunction in healthy individuals, rendering them more susceptible to acute coronary syndrome (Zupancic, 2009). For example, a recent study found an increased incidence in myocardial infarction cases during World Cup soccer matches (1998, 2002, 2006, 2010), although it did not affect in-hospital mortality rates (Guilherme *et al.*, 2013). Moreover, job strain - defined as high job demands and low decision latitude - is associated with increased hypertension incidence (Markovitz *et al.*, 2004; Guimont *et al.*, 2006). Here individuals with limited social support at the work place were at a relatively higher risk for developing hypertension (Guimont *et al.*, 2006). Differences based on gender, age and socioeconomic status appear to be relatively minor, hence indicating that the association between work strain and the development of cardiovascular complications is strong (Kivimäki and Kawachi, 2015).

Takotsubo cardiomyopathy - also known as ‘’broken heart syndrome’’ – is a stress-related complication that presents with ST-segment elevation, chest pain and increased creatine kinase and troponin levels (indicative of heart damage) (Szardien *et al.*, 2013). These symptoms are known to appear after physical or emotional stress (Komamura, 2014). The prevalence of takotsubo cardiomyopathy among

individuals with symptoms suggestive of acute coronary syndrome is ~0.7–2.5%, highlighting the similarity between acute coronary syndrome and takotsubo cardiomyopathy (Szardien *et al.*, 2013).

Throughout the literature there is evidence that psychological stress is associated with the onset of cardiovascular disease, specifically the induction of myocardial infarction (Ambrose and Singh, 2015; Kivimäki and Kawachi, 2015). However, the underlying mechanisms driving stress-related damage during myocardial ischemia and with reperfusion require further consideration.

4. Stress-induced myocardial ischemia and reperfusion damage

As discussed, myocardial infarction is a prominent feature of acute coronary syndrome. Myocardial infarction is the death of cardiomyocytes following an ischemic event in the myocardium (Davies, 1977; Norris, 1989; Hashmi and Al-Salam, 2015). How is stress linked to the onset of a myocardial infarction? An extensive systematic review of psychological stress-induced ischemia showed that psychological stress induces transient ischemia in humans (Strike and Steptoe, 2003). These responses were not due to severe emotional triggers, but rather behavioral challenges that could be encountered in everyday life (Strike and Steptoe, 2003). Others investigated the effects of mental stress at the time of acute myocardial infarction and found that patients experiencing moderate to high levels of stress are more prone to long-term adverse effects than patients experiencing lower levels (Arnold *et al.*, 2012).

The restoration of blood flow to the previously ischemic area is known to trigger further damage to the heart, a phenomenon referred to as ‘’lethal reperfusion injury’’ (Piper, Meuter and Schäfer, 2003; Eltzschig and Eckle, 2011; Vishwakarma *et al.*, 2017). The question therefore arises whether chronic psychological stress impacts or contributes to lethal reperfusion injury. Of note, a repeated intermittent stress study done on rats showed an increase in infarct size following ischemia and reperfusion (Scheuer and Mifflin, 2017). Table 1.1 summarizes animal studies that investigated the links between chronic

stress and ischemia-reperfusion, with the common theme that chronic stress resulted in an increased infarct size following ischemia and reperfusion.

Although the studies summarized in Table 1.1 clearly show a link between chronic stress and ischemia-reperfusion damage, the question remains regarding the underlying mechanisms driving this process. The focus of the rest of the review will be on inflammation and oxidative stress, and the potential role such mechanisms may play in the context of chronic stress and ischemia-reperfusion.

Table 1.1: Animal studies assessing the effect of chronic stress on ischemia-reperfusion injury.

Reference	Stress model	Duration of stress protocol	Ischemia/reperfusion model	Duration of I/R	Animal model	Findings
(Scheuer and Mifflin, 2017)	Restrain stress (1-1.5 hours a day)	8 – 14 days	*In vivo*	30' ischemia 180' reperfusion	Male Sprague Dawley rats	↑ infarct size 2/6 developed severe arrhythmia
	Restraint stress (2 hours a day)	11 – 12 days	*In vivo*	30' ischemia 180' reperfusion	Male Sprague Dawley rats	↑ infarct size 2/5 developed severe arrhythmia
(Rakhshan *et al.*, 2015)	Physical stress (Electric shock)	7 days	*Ex vivo* Langendorff	30' ischemia 60' reperfusion	Male Wistar rats	↑ infarct size
	Psychological stress (Witness physical stress)	7 days	*Ex vivo* Langendorff	30' ischemia 60' reperfusion	Male Wistar rats	↑ infarct size
(Ledvényiová-Farkašová *et al.*, 2015)	Crowding stress (70 cm^2 per 100g)	14 days	*Ex vivo* Langendorff	30' ischemia 120' reperfusion	Male Wistar Kyoto rats (5 weeks)	-
	Crowding stress (70 cm^2 per 100g)	14 days	*Ex vivo* Langendorff	30' ischemia 120' reperfusion	Female Wistar Kyoto rats (5 weeks)	↓ Ventricular tachycardia
	Crowding stress (70 cm^2 per 100g)	14 days	*Ex vivo* Langendorff	30' ischemia 120' reperfusion	Male SHR rats (5 weeks)	↑ Ventricular tachycardia

	Crowding stress (70 cm^2 per 100g)	14 days	*Ex vivo* Langendorff	30' ischemia 120' reperfusion	Female SHR rats (5 weeks)	↑ Ventricular tachycardia
(Ravingerová *et al.*, 2011)	Crowding stress (200 cm^2 per rat)	8 weeks	*Ex vivo* Langendorff	30' ischemia 120' reperfusion	Male Wistar Kyoto rats (Adult)	↑ duration of ventricular tachycardia ↓ LVDP recovery
	Crowding stress (200 cm^2 per rat)	8 weeks	*Ex vivo* Langendorff	30' ischemia 120' reperfusion	Male SHR rats (Adult)	↑ LVDP recovery ↓ duration of ventricular tachycardia
(Rorabaugh *et al.*, 2015)	Social instability Predator exposure (Day 1 and 11) (1 hour)	31 days	*Ex vivo*	20' ischemia 120' reperfusion	Male Sprague Dawley rats (Adult)	↑ infarct size
	Social instability Predator exposure (Day 1 and 11) (1 hour)	31 days	*Ex vivo*	20' ischemia 120' reperfusion	Female Sprague Dawley rats (Adult)	-

5. Inflammation as a link between chronic stress and ischemia-reperfusion injury

5.1. Chronic stress and inflammation

5.1.1. HPA-axis and inflammation

It is well known that activation of the HPA-axis under stressful situations leads to downstream production and secretion of cortisol from the adrenal glands (Tsigos and Chrousos, 2002; Stephens *et al.*, 2012; Agorastos *et al.*, 2018). Under normal conditions cortisol is a potent anti-inflammatory agent when bound to GR (Sorrells *et al.*, 2009; Hannibal and Bishop, 2014). However, under stressful conditions human plasma cortisol levels can increase ~10-fold above normal concentrations (Rogers *et al.*, 2015). GR resides in the cytoplasm in its inactive state where it associates with a complex of chaperone proteins including heat shock proteins (Juruena, 2014). After cortisol binds GR undergoes conformational changes and disassociates from this protein complex. GR then moves into the nucleus where it binds to DNA, i.e. targeting glucocorticoid response elements (GREs). GREs can either induce a positive or negative feedback on the genes it is linked to (Juruena, 2014). For example, glucocorticoids exert its anti-inflammatory effects by suppressing the expression of nuclear factor kappa-light chain enhancer of B-cells (NFκB) and activator protein-1 (AP-1) through GR binding (Cruz-Topete and Cidlowski, 2014). Glucocorticoids are known to induce apoptosis of T-lymphocytes, basophils, neutrophils and eosinophils to ultimately reduce inflammation (Sorrells and Sapolsky, 2007). Additionally, in a model of lipopolysaccharide (LPS)-induced sepsis, glucocorticoids can regulate cytokine production through inhibiting p38 mitogen-activated-protein-kinases (MAPK) (Bhattacharyya *et al.*, 2007; Busillo and Cidlowski, 2013).

However, long-term cortisol secretion induces a pro-inflammatory state (Hannibal and Bishop, 2014). For example, rats with higher plasma corticosterone levels display increased prostaglandin E2 (PGE_2)

accumulation (Pérez-Nievas *et al.*, 2007). The potential mechanisms underlying this likely include GR resistance, impaired negative feedback and ultimately cortisol depletion (Hannibal and Bishop, 2014). Chronic and excessive cortisol secretion may also result in GR resistance which then prevents cortisol from binding to the GR. This results in the failure of GR to downregulate inflammation (Cohen *et al.*, 2012). The impaired binding to GR disrupts the negative feedback system, thus CRF and cortisol continue to be released (Hannibal and Bishop, 2014). Such spikes in cortisol secretion may also increase its affinity for MR, which when bound to cortisol, elicits pro-inflammatory effects (Hannibal and Bishop, 2014). The elevated inflammatory byproducts may then damage the GR to thereby worsen cortisol dysfunction (Yang, Ray and Matthews, 2012). The continuous cortisol secretion induced by the inability of the negative feedback to maintain its secretion ultimately results in adrenal fatigue. In support, animal studies showed decreased cortisol levels following two weeks of repeated restraint stress (Fries *et al.*, 2005). Thus, even though cortisol is regarded as an anti-inflammatory agent at basal levels, it is clear that chronic activation of the HPA-axis induces a pro-inflammatory state through cortisol's inability to bind to GRs.

5.1.2. The autonomic nervous system and inflammation

As discussed, chronic activation of the HPA-axis induces a pro-inflammatory state. The autonomic nervous system also plays a vital role in the immune system. Here epinephrine and norepinephrine regulate the release of cytokines through α - and β-receptors on immune cells (Won and Kim, 2016). Studies show that norepinephrine increases tumor necrosis factor alpha (TNFα) production, while both epinephrine and norepinephrine stimulate the release of interleukin-6 (IL-6) (Bertini *et al.*, 1993; Chrousos, 2006). By contrast, acetylcholine is known to inhibit TNFα production along with other pro-inflammatory cytokines (IL-1 and IL-6) (Borovikova *et al.*, 2000). These results support the fact that sympathetic nervous system activation induces a pro-inflammatory state, while parasympathetic nervous system activation attenuates it. Figure 1.3 summarizes the effect of the autonomic nervous system on the cytokine production.

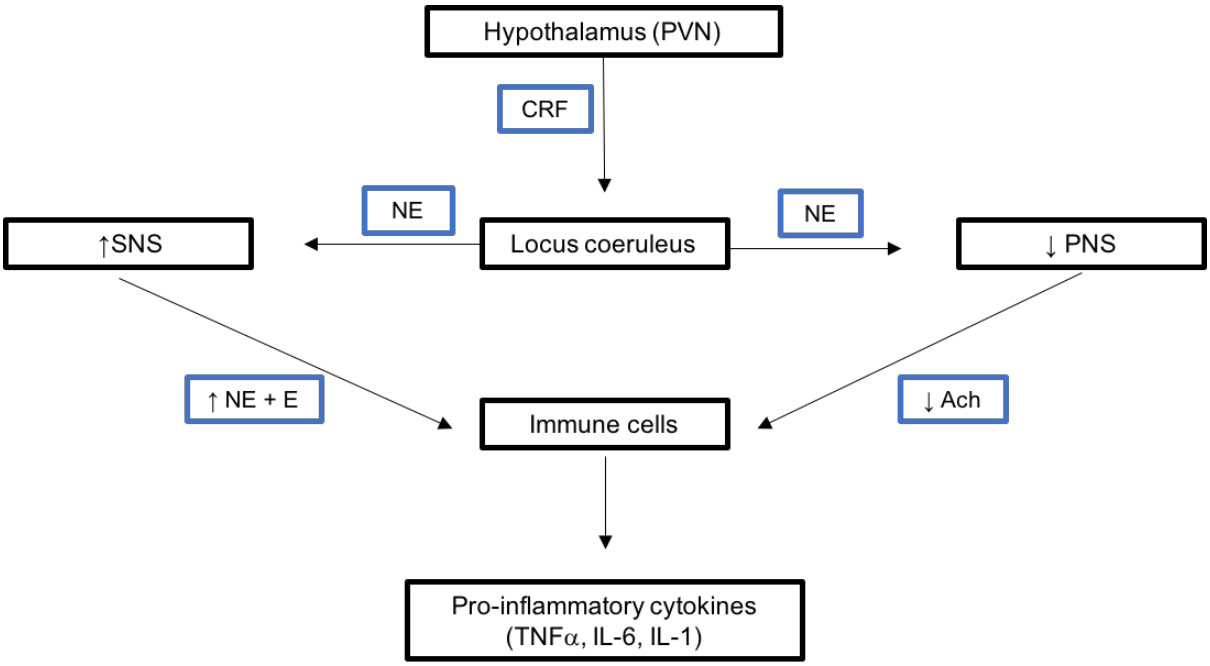


Figure 1.3: Links between the autonomic nervous and immune systems. CRF: Corticotropin releasing factor, NE: norepinephrine, E: epinephrine, Ach: acetylcholine, SNS: sympathetic nervous system, PNS: parasympathetic nervous system, PVN: paraventricular nucleus.

Activation of the HPA-axis and sympathetic nervous system are both involved in the stress response and studies showed that chronic activation of both systems can induce a pro-inflammatory state (Bertini *et al.*, 1993; Chrousos, 2006; Smith and Vale, 2006).

5.2. Ischemia-reperfusion and inflammation

The previous section covered the role that inflammation plays during times of chronic stress. The next question to consider is what role inflammation plays during ischemia-reperfusion and whether this could potentially be exacerbated by chronic stress-induced inflammation?

Inflammation constitutes a key component in the development of cardiovascular disease. IL-6 and C-reactive protein (CRP) are two important biomarkers indicative of systemic inflammation and the onset

of atherosclerosis (Nadrowski *et al.*, 2016; Y.-Z. Liu, Wang and Jiang, 2017). During ischemia-reperfusion, the inflammatory signals are generated by cardiomyocytes and endothelial cells (Vinten-Johansen, 2004). This sterile immune response involves activation of the innate, adaptive immune response as well as the complement system (Eltzschig and Eckle, 2011) (Figure 1.4).

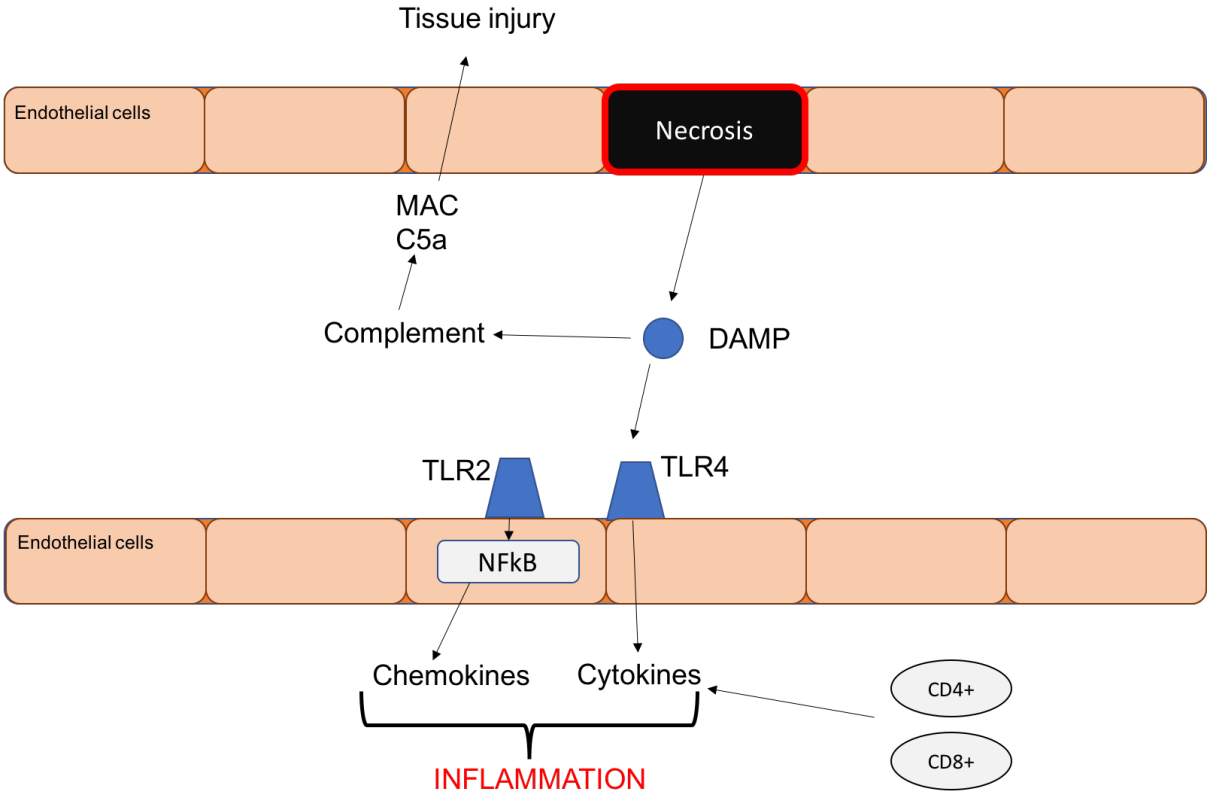


Figure 1.4: The inflammatory response following ischemia-reperfusion. DAMP: damage associated molecular patterns, MAC: membrane attack complex, NFκB: nuclear factor kappa-light chain enhancer of B-cells, TLR: toll-like receptors.

5.2.1. Innate immune response during ischemia-reperfusion

The necrotic myocardial tissue (infarct zone) and damaged extracellular matrix release damage-associated molecular patterns (DAMPs), that stimulate the complement cascade and also toll-like receptors (TLRs) resulting in the activation of NFκB and the subsequent stimulation of cytokines,

chemokines and cell-adhesion molecules (Fang *et al.*, 2015). Complement fragments, cytokines and neutrophil activating protein are released from the myocardium at the site of ischemia-reperfusion and perform as activating or chemoattractant factors that can induce neutrophilic events (Vakeva *et al.*, 1998; Dörge *et al.*, 2002). Although such an inflammatory response assists with wound healing, extreme inflammatory responses can cause left ventricular remodeling and heart failure (Fang *et al.*, 2015). The importance of inflammation in stress-related cardiac complications is supported by several studies. For example, TLR-4 deficient mice sustained smaller infarcts and decreased inflammation following acute myocardial infarction (Oyama *et al.*, 2004; Timmers *et al.*, 2008). Additionally, TLR-4 deletion reduced serum levels of TNFα, IL-6 and IL-1B (Kim *et al.*, 2007), highlighting the importance of TLR-4 in mediating local and systemic inflammatory response during ischemia-reperfusion. Cell adhesion molecules, like intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are strong indicators of the activation of endothelial cells, leukocytes and platelets (Fang *et al.*, 2015). Such circulating cell adhesion molecules, especially ICAM-1, are significantly related to future myocardial infarction or angina in healthy patients (Luc *et al.*, 2003).

mRNA profiling research showed that leukocytes (e.g. neutrophils and monocytes) are more active in the transcription of inflammatory genes in the blood of myocardial infarction patients (Wettinger *et al.*, 2005). Neutrophils play an important role in innate immunity and are known to infiltrate the infarcted area and mediate tissue damage by releasing reactive oxygen species and matrix-degrading enzymes (Vinten-Johansen, 2004; Fang *et al.*, 2015). Previous studies confirmed that a high neutrophil count was strongly related to adverse clinical outcomes in patients with acute coronary syndrome (Núñez *et al.*, 2008; Guasti *et al.*, 2011).

Monocytes are another leukocyte type that play an important role in atherosclerosis and acute coronary events, and are the most abundant immune cells in atherosclerotic plaques (Fang *et al.*, 2015). Moreover, studies showed that monocytosis is associated with left ventricular dysfunction after acute myocardial infarction (Maekawa *et al.*, 2002; Hong *et al.*, 2007).

5.2.2. Adaptive immune response during ischemia-reperfusion

T-lymphocytes play a crucial role in the induction of the adaptive inflammatory response following ischemia-reperfusion injury (Eltzschig and Eckle, 2011), e.g. they can accumulate at the infarct zone 24 hours after reperfusion (Schroeter *et al.*, 1994). In support, the inhibition of CD4+ T-lymphocyte accumulation and activation in the heart resulted in a decrease in infarct size following ischemia-reperfusion in mice (Yang *et al.*, in 2006). Furthermore, CD4 deficient mice were protected from ischemia-reperfusion injury in the liver (Zwacka *et al.*, 1997), highlighting the crucial role of CD4 cells in the process of ischemia-reperfusion.

5.2.3. Complement system during ischemia-reperfusion

The complement system acts as a surveillance system that can differentiate between healthy tissue, cellular debris and foreign matter and elicit an according response (Eltzschig and Eckle, 2011). Excessive complement activation is detrimental, although a threshold in terms of activation is important in tissue regeneration (He *et al.*, 2009). The necrosis induced by ischemia and reperfusion in endothelial cells results in the release of membrane constituents that are capable of triggering the complement system (Zuidema, 2010). This includes the release of complement factor 3a (C3a), complement factor 5a (C5a) and the cytolytic terminal membrane attack complex (MAC) (Arumugam *et al.*, 2004). Complement factor 5 (C5) is cleaved by C5 convertase to yield C5a and MAC. C5a is known as one of the most inflammatory peptides (Hugli, 1986). MAC can also stimulate the release of reactive oxygen species (ROS) from monocytes thereby inducing further damage (Arumugam *et al.*, 2004). The inhibition of C5 cleavage and hence the prevention of C5a and MAC formation can significantly reduce the degree of damage following ischemia-reperfusion (Wada, Montalto and Stahl, 2001).

5.2.4. Pro-inflammatory cytokines

Pro-inflammatory cytokines play an important role in the induction of the innate and adaptive immune responses. This happens through the stimulation of chemokines and adhesion molecules (Fang *et al.*, 2015). CRP is a well-known predictor of mortality in patients with acute coronary syndrome (Sheikh *et al.*, 2012). Additionally, IL-6 also correlates with CRP levels as it is the main stimulus for CRP production in the liver (Dinarello, 2011).

Even though the heart is under distress, other organs also play a role in the inflammatory response that contribute to the lethal reperfusion damage. Following myocardial infarction, monocytopoiesis in the spleen yields an increased monocyte production which could contribute to myocardial infarction through inducing leukocyte influx into atherosclerotic plaques (Leuschner *et al.*, 2012; Fang *et al.*, 2015).

As summarized in Figure 1.4, inflammation plays a major role in aggravating damage following reperfusion. The adaptive and innate immune responses both upregulate pro-inflammatory cytokine and chemokine production, while the complement system can further induce damage through MAC (Arumugam *et al.*, 2004).

To summarize this section, chronic psychological stress induces a pro-inflammatory state through activation of the HPA-axis and sympathetic nervous system (Bertini *et al.*, 1993; Chrousos, 2006; Cohen *et al.*, 2012). A pro-inflammatory state that could potentially aggravate the ischemia-reperfusion induced injury (Figure 1.5).

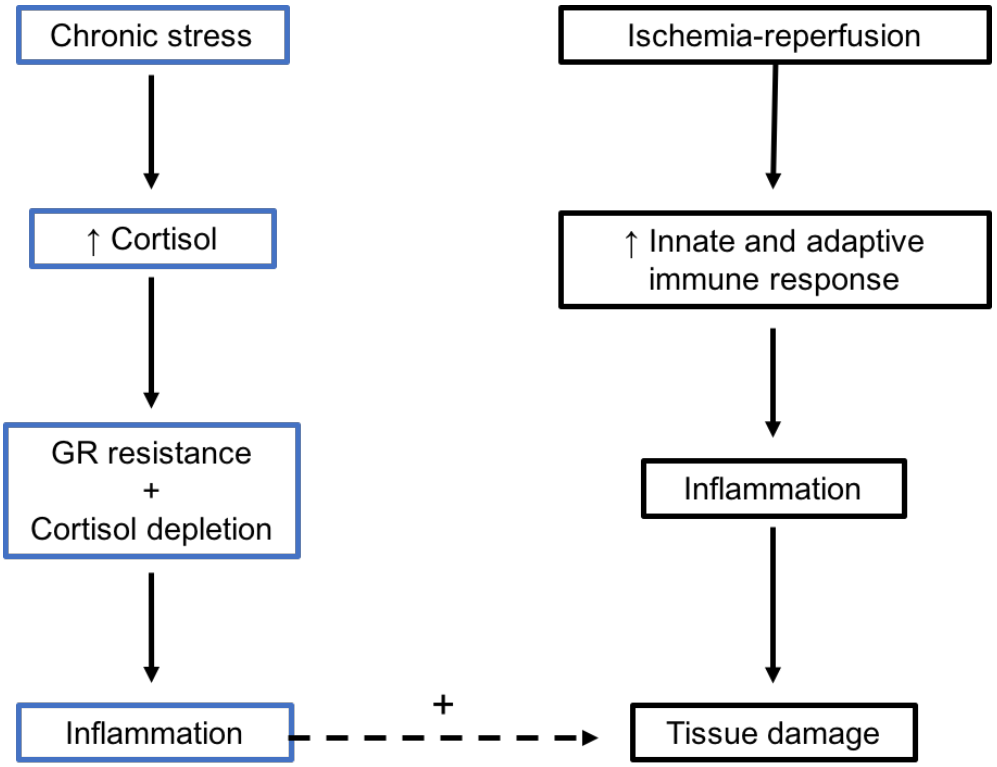


Figure 1.5: Inflammation as a potential mechanism whereby chronic psychological stress renders the heart more susceptible to ischemia-reperfusion injury. GR: glucocorticoid receptor

6. Oxidative stress as a link between chronic stress and ischemia-reperfusion injury

The second focus of this review is oxidative stress and the role it plays in chronic psychological stress and ischemia-reperfusion injury.

6.1. Chronic stress and oxidative stress

Psychological stress is associated with increased levels of oxidative stress (Aschbacher *et al.*, 2013). For example, stress-mediated glucocorticoid-induced neurodegeneration may be caused by increased ROS production (Zafir and Banu, 2009). Moreover, others showed increased serum levels of 8-

hydroxy-2'-deoxyguanosine - a known biomarker for oxidative damage - in patients with depression (Forlenza and Miller, 2006).

Cortisol is a potential link between stress and oxidative stress (Aschbacher *et al.*, 2013). In support, rats experiencing chronic stress or treated with corticosterone displayed decreased superoxide dismutase (SOD) and catalase activity (Zafir and Banu, 2009). Others found a strong correlation between the excretion of cortisol and oxidative markers in urine (Joergensen *et al.*, 2011). Cortisol displays a strong inverted "U"-shaped relationship with mitochondrial function, an important role player in oxidative stress (Du *et al.*, 2009). Brief high doses of cortisol improved mitochondrial function in cell culture models, while chronic high doses of cortisol dramatically decrease mitochondrial function and promoted cell death (Du *et al.*, 2009). Another way whereby cortisol may potentially induce oxidative injury is by damaging intracellular constituents such as DNA and RNA (Gidron *et al.*, 2006). In support, Gidron and colleagues reported a strong correlation between psychological factors (e.g. depression) and DNA-damage in humans (Gidron *et al.*, 2006).

As discussed, activation of the sympathetic nervous system by chronic stress leads to increased pro-inflammatory cytokine production (Figure 1.3) (Won and Kim, 2016). These cytokines can enhance the activity of indoleamine 2,3-dioxygenase (IDO), which is the main enzyme responsible for converting tryptophan to kynurenine (Won and Kim, 2016). As indicated in Figure 1.6, downstream metabolites of this reaction (3-hydroxykynurenine and 3-hydroxyanthranilic) are known to induce oxidative damage in the brain (Fukui *et al.*, 1991; Vazquez *et al.*, 2000).

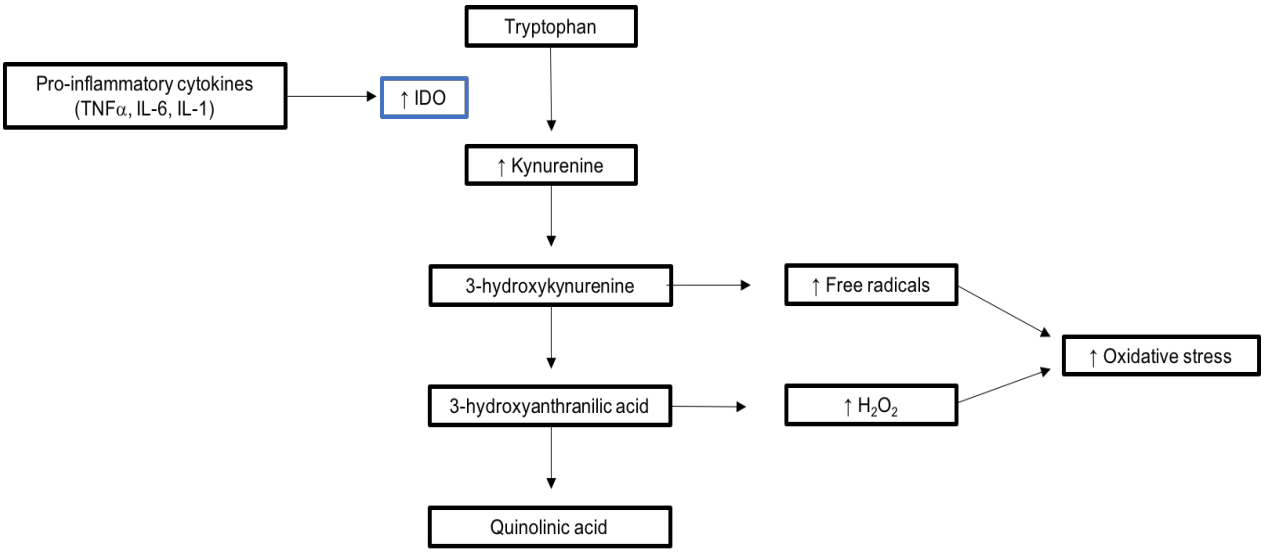


Figure 1.6: Link between sympathetic nervous system-induced inflammation and oxidative stress. IDO: indoleamine 2,3-dioxygenase.

6.2. Ischemia-reperfusion and oxidative stress

The increase in reoxygenation to the previously ischemic tissue is accompanied by a burst of ROS which plays a significant role in myocardial reperfusion injury (Yellon and Hausenloy, 2007; González-Montero *et al.*, 2018). Examples of ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and peroxynitrate anion ($ONOO^-$) (González-Montero *et al.*, 2018). Oxidative stress forms part of the oxygen paradox where reoxygenation induces a degree of damage that exceeds that induced by the ischemia itself (Yellon and Hausenloy, 2007). The oxidative stress induced during reperfusion also decreases the bioavailability of nitric oxide, thus diminishing its cardioprotective effects (Yellon and Hausenloy, 2007). Xanthine oxidase (XO), uncoupled eNOS and NADPH oxidases (NOX) are the most important sources of ROS within the myocardium (Cadenas, 2018; González-Montero *et al.*, 2018). Activation of NOX results in a one-electron reduction of oxygen to then generate the superoxide anion. NADPH oxidases are mainly present in activated neutrophils where they generate large amounts of superoxide (Brandes and Kreuzer, 2005; González-Montero *et al.*, 2018). The damaging effects of NOX were emphasized by NOX-1 and NOX-2 knockout mice that displayed significantly attenuated

myocardial reperfusion injury compared to control mice (Braunersreuther *et al.*, 2013). However, they found no protective effects during the ischemic phase of the *ex vivo* protocol, suggesting the damage induced by NOX-1 and NOX-2 occurs during reperfusion (Braunersreuther *et al.*, 2013).

Xanthine oxidoreductase is crucial in the hydroxylation of xanthine to uric acid. This enzyme occurs in two interconvertible forms, xanthine dehydrogenase and XO, with the former being found predominantly in healthy tissue (Granger and Kvietys, 2015). During ischemia adenosine triphosphate (ATP) is catabolized to hypoxanthine. Additionally, xanthine dehydrogenase is converted to XO (Figure 1.7). Upon restoration of blood flow, there is an increased supply of oxygen availability, and oxygen reacting with hypoxanthine and XO to generate superoxide and hydrogen peroxide (Figure 1.7) (Granger and Kvietys, 2015). Despite there being evidence for the post-ischemic protective effects of XO inhibitors, other studies failed to report any protection following inhibition of XO (Downey *et al.*, 1987; Metzger and Lauterburg, 1988). However, this is dependent on the baseline activity of XO, e.g. there is almost no XO expression in rabbit hearts (Downey *et al.*, 1987) as is the case for human hearts (Eddy *et al.*, 2017).

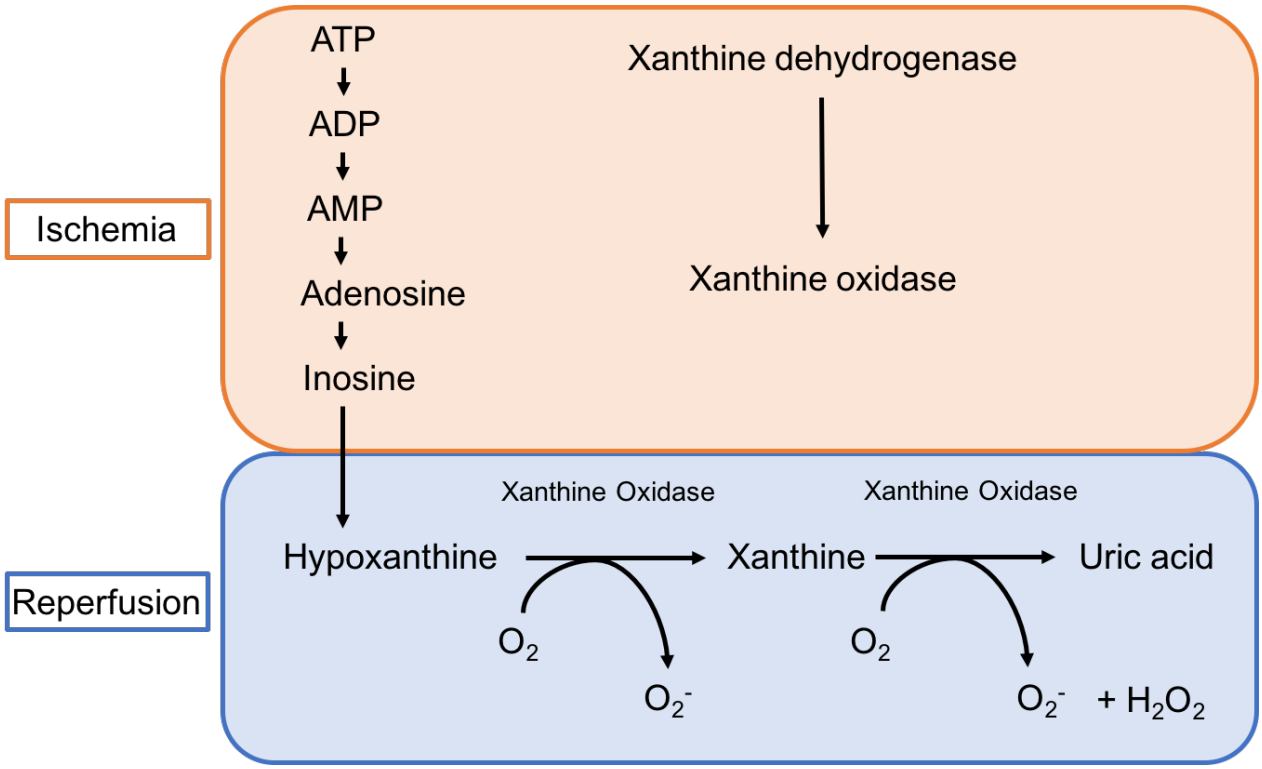


Figure 1.7: The role of xanthine oxidase in the production of ROS during ischemia and reperfusion.

ATP: adenosine triphosphate, ADP: adenosine diphosphate, AMP: adenosine monophosphate

Superoxide dismutase catalyzes the dismutation of superoxide to hydrogen peroxide, with hydrogen peroxide thereafter reduced to water and oxygen (Dhalla *et al.*, 2000). Activity and gene expression of MnSOD, catalase and glutathione peroxidase were significantly increased in hearts experiencing four cycles of ischemia and reperfusion, compared to those subjected to only one cycle (Das, Engelman and Kimura, 1993). Additionally, others found that overexpression of MnSOD rendered the heart more resistant to ischemia-reperfusion injury (Chen *et al.*, 1998), therefore emphasizing the importance of anti-oxidants during ischemia and reperfusion.

The role and expression of SOD, NOX and XO during ischemia-reperfusion emphasizes the importance of oxidative stress within the context of ischemia and reperfusion. How does this link with psychological stress? As mentioned above it is well known that oxidative stress is an important component in reperfusion injury (Yellon and Hausenloy, 2007; González-Montero *et al.*, 2018) and is also a distinguishing feature during chronic psychological stress (Aschbacher *et al.*, 2013; Won and

Kim, 2016). This could indicate that the pro-oxidative state induced by chronic psychological stress may contribute to the oxidative stress induced during ischemia-reperfusion, and ultimately exacerbate the damage.

7. Conclusion

It is becoming evident that chronic psychological stress is a serious risk factor for the development of cardiovascular disease (Yusuf *et al.*, 2004). It is clear that both ischemia-reperfusion injury and chronic stress are characterized by a robust inflammatory response together with the induction of oxidative stress (Figure 1.8). In support, numerous studies found that chronic stress does indeed exacerbate ischemia-reperfusion injury (Table 1.1). However, the exact mechanisms linking stress and ischemia-reperfusion injury require further investigation to ascertain whether it is actually stress-related inflammation and oxidative stress that renders the heart more susceptible to the reperfusion damage.

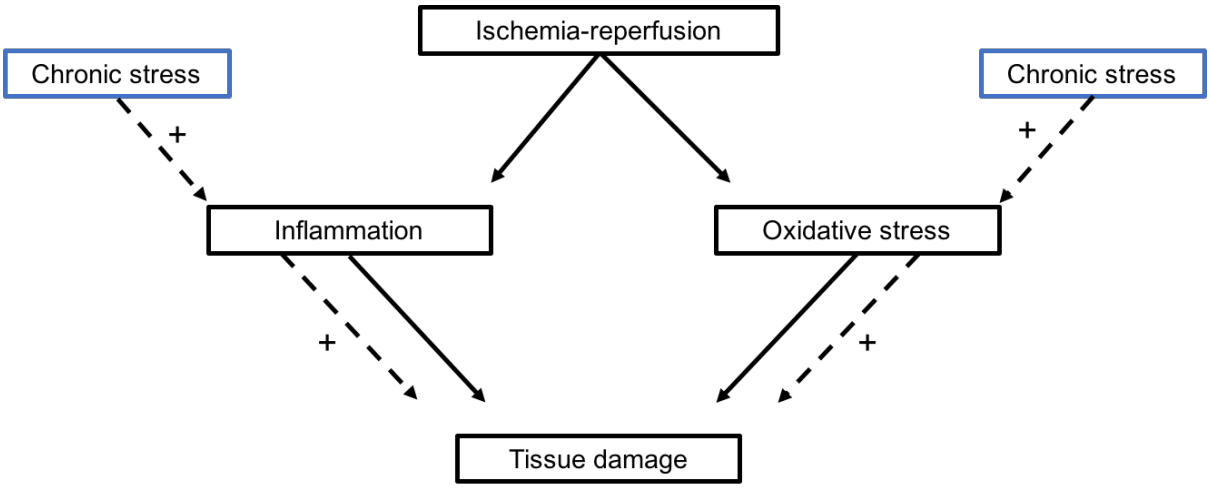


Figure 1.8: Potential mechanisms involved in chronic psychological stress exacerbating ischemia-reperfusion injury. Inflammation and oxidative stress both contribute to ischemia-reperfusion injury. Through chronic activation of the HPA-axis, cortisol's anti-inflammatory properties are diminished. Additionally, persistent upregulation of the sympathetic nervous system and downregulation of the parasympathetic nervous system result in the production of pro-inflammatory cytokines. Together the pro-

inflammatory and pro-oxidative state induced by chronic stress may potentially aggravate ischemia-reperfusion-related injury.

8. References

1) Abdullah, K. (2016) *WORLD'S MOST STRESSED COUNTRIES – RANKED*. Available at: https://www.atlasandboots.com/most-stressed-countries/.
2) Agorastos, A., Pervanidou, P., Chrousos, G. and Kolaitis, G. (2018) 'Early life stress and trauma: developmental neuroendocrine aspects of prolonged stress system dysregulation', *Hormones*. Hormones, 17(4), pp. 507–520. doi: 10.1007/s42000-018-0065-x.
3) Ambrose, J. A. and Singh, M. (2015) 'Pathophysiology of coronary artery disease leading to acute coronary syndromes.', *F1000prime reports*, 7, p. 08. doi: 10.12703/P7-08.
4) Arnold, S. V. Smolderen, K., Buchanan, D., Li, Y. and Spertus, J. (2012) 'Perceived stress in myocardial infarction: Long-term mortality and health status outcomes', *Journal of the American College of Cardiology*. doi: 10.1016/j.jacc.2012.06.044.
5) Arumugam, T. V., Shiels, I., Woodruff, T., Granger, D. and Taylor, S. (2004) 'The role of the complement system in ischemia-reperfusion injury.', *Shock (Augusta, Ga.)*. doi: 10.1097/00024382-200405000-00002.ISSN: 00192805
6) Aschbacher, K. O'Donovan, A., Wolkowitz, O., Dhabhar, F., Su, Y. and Epel, E. (2013) 'Good stress, bad stress and oxidative stress: Insights from anticipatory cortisol reactivity', *Psychoneuroendocrinology*. doi: 10.1016/j.psyneuen.2013.02.004.
7) Bertini, R., Garattini, S., Delgado, R. and Ghezzi, P. (1993) 'Pharmacological activities of chlorpromazine involved in the inhibition of tumour necrosis factor production in vivo in mice', *Immunology*.
8) Bhattacharyya, S., Brown, D., Brewer, J., Vogt, S. and Muglia, L. (2007) 'Macrophage glucocorticoid receptors regulate Toll-like receptor 4-mediated inflammatory responses by selective inhibition of p38 MAP kinase', *Blood*. doi: 10.1182/blood-2006-10-048215.
9) Bloomberg (2013) *Most stressed-out: Countries*. Available at: https://www.bloomberg.com/graphics/best-and-worst/#most-stressed-out-countries

(Accessed: 12 July 2019).

10) Borovikova, L., Ivanova, S., Zhang, M., Yang, H., Botchkina, G., Watkins, L., Wang, H., Abumrad, N., Eaton, J. and Tracey, K. (2000) 'Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin', *Nature*. doi: 10.1038/35013070.

11) Brandes, R. P. and Kreuzer, J. (2005) 'Vascular NADPH oxidases: molecular mechanisms of activation.', *Cardiovascular research*. doi: 10.1016/j.cardiores.2004.08.007.

12) Braunersreuther, V., Montecucco, F., Asrih, M., Pelli, G., Galan, K., Frias, M., Burger, F., Quindere, A., Montessuit, C., Krauses, K., Mach, F. and Jaquet, V. (2013) 'Role of NADPH oxidase isoforms NOX1, NOX2 and NOX4 in myocardial ischemia/reperfusion injury', *Journal of Molecular and Cellular Cardiology*. doi: 10.1016/j.yjmcc.2013.09.007.

13) Busillo, J. M. and Cidlowski, J. A. (2013) 'The five Rs of glucocorticoid action during inflammation: Ready, reinforce, repress, resolve, and restore', *Trends in Endocrinology and Metabolism*. doi: 10.1016/j.tem.2012.11.005.

14) Cadenas, S. (2018) 'ROS and redox signaling in myocardial ischemia-reperfusion injury and cardioprotection', *Free Radical Biology and Medicine*. doi: 10.1016/j.freeradbiomed.2018.01.024.

15) Chen, Z., Siu, B., Ho, Y., Vincent, R., Chua, C., Hamdy, R. and Chua, B. (1998) 'Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice', *Journal of Molecular and Cellular Cardiology*. doi: 10.1006/jmcc.1998.0789.

16) Chrousos, G. P. (2009) 'Stress and disorders of the stress system', *Nature Reviews Endocrinology*. doi: 10.1038/nrendo.2009.106.

17) CHROUSOS, G. P. (2006) 'The Stress Response and Immune Function: Clinical Implications: The 1999 Novera H. Spector Lecture', *Annals of the New York Academy of Sciences*. doi: 10.1111/j.1749-6632.2000.tb05371.x.

18) Chrousos, G. P. and Gold, P. W. (1992) 'The Concepts of Stress and Stress System Disorders: Overview of Physical and Behavioral Homeostasis', *JAMA: The Journal of the American Medical*

Association. doi: 10.1001/jama.1992.03480090092034.

19) Cohen, S., Janicki-Deverts, D., Doyle, W., Miller, G., Frank, E., Rabin, B. S. and Turner, R. (2012) 'Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk.', *Proceedings of the National Academy of Sciences of the United States of America*, 109(16), pp. 5995–9. doi: 10.1073/pnas.1118355109.

20) Cruz-Topete, D. and Cidlowski, J. A. (2014) 'One hormone, two actions: Anti- And pro-inflammatory effects of glucocorticoids', *NeuroImmunoModulation*. doi: 10.1159/000362724.

21) Das, D. K., Engelman, R. M. and Kimura, Y. (1993) 'Molecular adaptation of cellular defences following preconditioning of the heart by repeated ischaemia', *Cardiovascular Research*. doi: 10.1093/cvr/27.4.578.

22) Davies, M. J. (1977) 'The pathology of myocardial ischaemia', *Journal of Clinical Pathology*, (S3-11), pp. 45–52. doi: 10.1136/jcp.s3-11.1.45.

23) Dhalla, N. S., Elmoselhi, A.B., Hata, T. and Makino, N. (2000) 'Status of myocardial antioxidants in ischemia-reperfusion injury', *Cardiovascular Research*. doi: 10.1016/S0008-6363(00)00078-X.

24) Dinarello, C. A. (2011) 'A clinical perspective of IL-1β as the gatekeeper of inflammation', *European Journal of Immunology*. doi: 10.1002/eji.201141550.

25) Dörge, H., Schulz, R., Belosjorow, S., Post, H., van de Sand., Konietzka, I., Frede, S., Hartung, T., Vinten-Johansen, J., Youker, K., Entman, M., Erbel, R. and Heusch, G. (2002) 'Coronary microembolization: The role of TNF-α in contractile dysfunction', *Journal of Molecular and Cellular Cardiology*. doi: 10.1006/jmcc.2001.1489.

26) Downey, J. M., Miura, T., Eddy, L. J., Chamber, D. E., Mellert, T., Hearse, D. J. and Yellon, D. M. (1987) 'Xanthine oxidase is not a source of free radicals in the ischemic rabbit heart', *Journal of Molecular and Cellular Cardiology*. doi: 10.1016/S0022-2828(87)80350-4.

27) Du, J., Wang, Y., Hunter, R., Wei, Y., Blumenthal, R., Falke, C., Khairova, R., Zhou, R., Yuan, P., Machado-Vieira, R., McEwen, B. S. and Manji, H. (2009) 'Dynamic regulation of mitochondrial

function by glucocorticoids', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0812671106.

28) Duric, V., Clayton, S., Leong, M. L. and Yuan, L. (2016) 'Comorbidity Factors and Brain Mechanisms Linking Chronic Stress and Systemic Illness', *Neural Plasticity*. doi: 10.1155/2016/5460732.

29) Eddy, L. J., Stewart, J. R., Jones, H. P., Engerson, T. D., McCord, J. M. and Downey, J. M. (2017) 'Free radical-producing enzyme, xanthine oxidase, is undetectable in human hearts', *American Journal of Physiology-Heart and Circulatory Physiology*. doi: 10.1152/ajpheart.1987.253.3.h709.

30) Eltzschig, H. K. and Eckle, T. (2011) 'Ischemia and reperfusion-from mechanism to translation', *Nature Medicine*. doi: 10.1038/nm.2507.

31) Fang, L., Moore, X. L., Dart, A. M. and Wang, L. M. (2015) 'Systemic inflammatory response following acute myocardial infarction', *Journal of Geriatric Cardiology*. doi: 10.11909/j.issn.1671-5411.2015.03.020.

32) Forlenza, M. J. and Miller, G. E. (2006) 'Increased serum levels of 8-hydroxy-2???-deoxyguanosine in clinical depression', *Psychosomatic Medicine*. doi: 10.1097/01.psy.0000195780.37277.2a.

33) Fries, E., Hesse, J., Hellhammer, J. and Hellhammer, D. H. (2005) 'A new view on hypocortisolism', *Psychoneuroendocrinology*. doi: 10.1016/j.psyneuen.2005.04.006.

34) Fukui, S., Schwarcz, R., Rapoport, S. I., Takada, Y. and Smith, Q. R. (1991) 'Blood–Brain Barrier Transport of Kynurenines: Implications for Brain Synthesis and Metabolism', *Journal of Neurochemistry*. doi: 10.1111/j.1471-4159.1991.tb03460.x.

35) Gaffey, A. E., Bergeman, C. S., Clark, L. A. and Wirth, M. M. (2016) 'Aging and the HPA axis: Stress and resilience in older adults', *Neuroscience and Biobehavioral Reviews*. Elsevier Ltd, 68, pp. 928–945. doi: 10.1016/j.neubiorev.2016.05.036.

36) Gidron, Y., Russ, K., Tissarchondou, H. and Warner, J. (2006) 'The relation between

psychological factors and DNA-damage: A critical review', *Biological Psychology*. doi: 10.1016/j.biopsycho.2005.11.011.

37) Golbidi, S., Frisbee, J. C. and Laher, I. (2015) 'Chronic stress impacts the cardiovascular system: animal models and clinical outcomes', *American Journal of Physiology-Heart and Circulatory Physiology*. doi: 10.1152/ajpheart.00859.2014.

38) Goldstein, D. S. and Kopin, I. J. (2007) 'Evolution of concepts of stress', *Stress*. doi: 10.1080/10253890701288935.

39) González-Montero, J., Brito, R., Gajardo, A. and Rodrigo, R. (2018) 'Myocardial reperfusion injury and oxidative stress: Therapeutic opportunities', *World Journal of Cardiology*. doi: 10.4330/wjc.v10.i9.74.

40) Granger, D. N. and Kvietys, P. R. (2015) 'Reperfusion injury and reactive oxygen species: The evolution of a concept', *Redox Biology*. doi: 10.1016/j.redox.2015.08.020.

41) Guasti, L., Dentali, F., Castiglioni, L., Maroni, L., Marino, F., Squizzato, A., Ageno, W., Gianni, M., Gaudio, G., Grandi, A. M., Cosentino, M. and Venco, A. (2011) 'Neutrophils and clinical outcomes in patients with acute coronary syndromes and/or cardiac revascularization: A systematic review on more than 34,000 subjects', *Thrombosis and Haemostasis*. doi: 10.1160/TH11-02-0096.

42) Borges, D. G., Monteiro, R. A., Schmidt, A. and Pazin-Filho, A. (2013) 'World Soccer Cup as a Trigger of Cardiovascular Events', pp. 546–552. doi: 10.5935/abc.20130105.

43) Guimont, C., Brisson, C., Dagenais, G. R., Milot, A., Vezina, M., Masse, B., Moisan, J., Laflamme, N. and Blanchette, C. (2006) 'Effects of job strain on blood pressure: A prospective study of male and female white-collar workers', *American Journal of Public Health*. doi: 10.2105/AJPH.2004.057679.

44) Hannibal, K. E. and Bishop, M. D. (2014) 'Chronic Stress, Cortisol Dysfunction, and Pain: A Psychoneuroendocrine Rationale for Stress Management in Pain Rehabilitation', *Physical Therapy*. doi: 10.2522/ptj.20130597.

45) Hashmi, S. and Al-Salam, S. (2015) 'Acute myocardial infarction and myocardial ischemia-reperfusion injury: a comparison.', *International journal of clinical and experimental pathology*. PMID: 26464621

46) He, S., Atkinson, C., Qiao, F., Cianflone, K., Chen, X. and Tomlinson, S. (2009) 'A complement-dependent balance between hepatic ischemia/reperfusion injury and liver regeneration in mice', *Journal of Clinical Investigation*. doi: 10.1172/JCI38289.

47) Hill, M. N. Hellemans, K. G., Verma, P., Gorzalka, B. B. and Weinberg, J. (2012) 'Neurobiology of chronic mild stress: Parallels to major depression', *Neuroscience and Biobehavioral Reviews*. doi: 10.1016/j.neubiorev.2012.07.001.

48) Hong, Y. J., Jeong, M. H., Ann, Y., Yoon, N. S., Lee, S. R., Hong, S. N., Moon., J. Y., Kim. K. H., Park, H. W., Kim, J. H., Cho, J. G., Park, J. C. and Kang, J. C. (2007) 'Relationship Between Peripheral Monocytosis and Nonrecovery of Left Ventricular Function in Patients With Left Ventricular Dysfunction Complicated With Acute Myocardial Infarction', *Circulation Journal*. doi: 10.1253/circj.71.1219.

49) Hugli, T. E. (1986) 'Biochemistry and biology of anaphylatoxins', *Complement*. doi: 10.1159/000467889.

50) Joergensen, A., Broedbaek, K., Weimann, A., Semba, R. D., Ferrucci, L., Joergensen, M. and Poulsen, H. (2011) 'Association between urinary excretion of cortisol and markers of oxidatively damaged DNA and RNA in humans', *PLoS ONE*. doi: 10.1371/journal.pone.0020795.

51) Juruena, M. F. (2014) 'Early-life stress and HPA axis trigger recurrent adulthood depression', *Epilepsy and Behavior*. Elsevier Inc., 38, pp. 148–159. doi: 10.1016/j.yebeh.2013.10.020.

52) Kalogeris, T., Baines, C. P., Krenz, M. and Korthuis, R. (2014) *Cell Biology of Ischemia/Reperfusion Injury*. doi: 10.1016/B978-0-12-394309-5.00006-7.Cell.

53) Kim, S. C., Ghanem, A., Stapel, H., Tiemann, K., Knuefermann, P., Hoeft, A., Meyer, R., Grohe, C., Knowlton, A. A. and Baumgarten, G. (2007) 'Toll-like receptor 4 deficiency: Smaller infarcts, but no gain in function', *BMC Physiology*. doi: 10.1186/1472-6793-7-5.

54) Kivimäki, M. and Kawachi, I. (2015) 'Work Stress as a Risk Factor for Cardiovascular Disease', *Current Cardiology Reports*. doi: 10.1007/s11886-015-0630-8.

55) Komamura, K. (2014) 'Takotsubo cardiomyopathy: Pathophysiology, diagnosis and treatment', *World Journal of Cardiology*. doi: 10.4330/wjc.v6.i7.602.

56) Kumar, A. and Cannon, C. P. (2009) 'Acute coronary syndromes: Diagnosis and management, part I', in *Mayo Clinic Proceedings*. doi: 10.4065/84.10.917.

57) Ledvényiová-Farkašová, V., Bernatova, I., Balis, P., Puzserova, A., Bartekova, M., Gablovsky, I. and Ravingerova, T. (2015) 'Effect of crowding stress on tolerance to ischemia–reperfusion injury in young male and female hypertensive rats: molecular mechanisms', *Canadian Journal of Physiology and Pharmacology*, 93(9), pp. 793–802. doi: 10.1139/cjpp-2015-0026.

58) Leuschner, F., Rauch, P. J., Ueno, T., Gorbatov, R., Marinelli, B., Lee, W. W., Dutta, P., Wei, Y., Robbins, C., Iwamotto, Y., Sena, B., Chudnovskiy, A., Panizzi, P., Keliher, E., Higgins, J. M., Libby, P., Moskowitz, M. A., Pillet, M. J., Swirsky, F. K., Weissleder, R. and Nahremdorf, M. (2012) 'Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis.', *The Journal of experimental medicine*. doi: 10.1084/jem.20111009.

59) Lewis, D. I. and Coote, J. H. (1990) 'Excitation and inhibition of rat sympathetic preganglionic neurones by catecholamines', *Brain Research*. doi: 10.1016/0006-8993(90)91287-Q.

60) Liu, Y.-Z., Wang, Y.-X. and Jiang, C.-L. (2017) 'Inflammation: The Common Pathway of Stress-Related Diseases', *Frontiers in Human Neuroscience*. doi: 10.3389/fnhum.2017.00316.

61) Luc, G., Arveiler, D., Evans, A., Amouyel, P., Ferrieres, J., Bard, J. M., Elkhalil, L., Fruchart, J. C. and Ducimetiere, P. (2003) 'Circulating soluble adhesion molecules ICAM-1 and VCAM-1 and incident coronary heart disease: The PRIME Study', in *Atherosclerosis*. doi: 10.1016/S0021-9150(03)00280-6.

62) Maekawa, Y., Anzai, T., Yoshikawa, T., Asakura, Y., Takahashi, T., Ishikawa, S., Mitamura, H. and Ogawa, S. (2002) 'Prognostic significance of peripheral monocytosis after reperfused acute myocardial infarction: A possible role for left ventricular remodeling', *Journal of the American*

College of Cardiology. doi: 10.1016/S0735-1097(01)01721-1.

63) Markovitz, J. H., Matthews, K. A., Whooley, M., Lewis, C. E. and Greenlund, K. J. (2004) 'Increases in job strain are associated with incident hypertension in the CARDIA study', *Annals of Behavioral Medicine*. doi: 10.1207/s15324796abm2801_2.

64) Maydych, V. (2019) 'The interplay between stress, inflammation, and emotional attention: Relevance for depression', *Frontiers in Neuroscience*. doi: 10.3389/fnins.2019.00384.

65) Metzger, J. and Lauterburg, B. H. (1988) 'Effect of allopurinol on oxidant stress and hepatic function following ischemia and reperfusion in the rat', *Liver*. doi: 10.1111/j.1600-0676.1988.tb01014.x.

66) Nadrowski, P., Chudek, J., Skrzypek, M., Puzianowksa-Kuznicka, M., Mossakowska, M., Wiecek, A., Zdrojewski, T., Grodzicki, T. and Kozakiewicz, K. (2016) 'Associations between cardiovascular disease risk factors and IL-6 and hsCRP levels in the elderly', *Experimental Gerontology*. doi: 10.1016/j.exger.2016.10.001.

67) Nicolaides, N. C., Kyratzi, E., Lamprokostopoulou, A., Chrousos, G. P. and Charmandari, E. (2015) 'Stress, the Stress System and the Role of Glucocorticoids', *Neuroimmunomodulation*, 22(1–2), pp. 6–19. doi: 10.1159/000362736.

68) Norris, R. M. (1989) 'The pathophysiology of myocardial ischaemia', *Bailliere's Clinical Anaesthesiology*. doi: 10.1016/S0950-3501(89)80028-5.

69) Núñez, J., Nunez, E., Bodi, V., Sanchis, J., Minana, G., Mainar, L., Santas, E., Merlos, P., Rumiz, E., Darmofal, H., Heatta, A. M. and Llacer, A. (2008) 'Usefulness of the Neutrophil to Lymphocyte Ratio in Predicting Long-Term Mortality in ST Segment Elevation Myocardial Infarction', *American Journal of Cardiology*. doi: 10.1016/j.amjcard.2007.11.004.

70) Oyama, J. I., Blais, C., Liu, X., Pu, M., Kobzik, L, Kelly, R. A. and Bourcier, T. (2004) 'Reduced Myocardial Ischemia-Reperfusion Injury in Toll-Like Receptor 4-Deficient Mice', *Circulation*. doi: 10.1161/01.CIR.0000112575.66565.84.

71) Pérez-Nievas, B. G., Garcia-Bueno, B., Caso, J. R., Menchen, L. and Leza, J. C. (2007)

'Corticosterone as a marker of susceptibility to oxidative/nitrosative cerebral damage after stress exposure in rats', *Psychoneuroendocrinology*. doi: 10.1016/j.psyneuen.2007.04.011.

72) Piper, H. M., Meuter, K. and Schäfer, C. (2003) 'Cellular mechanisms of ischemia-reperfusion injury', in *Annals of Thoracic Surgery*. doi: 10.1016/S0003-4975(02)04686-6.

73) Rakhshan, K., Imani, A., Faghihi, M., Nabavizadeh, F., Golnazari, M. and Karimian, S. (2015) 'Evaluation of chronic physical and psychological stress induction on cardiac ischemia / reperfusion injuries in isolated male rat heart: The role of sympathetic nervous system', *Acta Medica Iranica*, 53(8), pp. 482–490.

74) Ravingerová, T., Bernatova, I., Matejikova, J., Ledvenyiova, V., Nemcekova, M., Pechanova, O., Tribulova, N. and Slezak, J. (2011) 'Impaired cardiac ischemic tolerance in spontaneously hypertensive rats is attenuated by adaptation to chronic and acute stress', *Experimental and Clinical Cardiology*. ISSN: 12056626

75) Rogers, K. M., Bonar, C. A., Estrella, J. L. and Yang, S. (2015) 'Inhibitory effect of glucocorticoid on coronary artery endothelial function', *American Journal of Physiology-Heart and Circulatory Physiology*. doi: 10.1152/ajpheart.00364.2002.

76) Rorabaugh, B.R.Krivenko, A., Eisenmann, E. D., Bui, A. D., Seeley, S., Fry, M.E, Lawson, J. D., Stoner, L. E., Johnson, B. L. and Zoladz, P. R. (2015) 'Sex-dependent effects of chronic psychosocial stress on myocardial sensitivity to ischemic injury', *Stress*, 18(6), pp. 645–653. doi: 10.3109/10253890.2015.1087505.

77) Salim, S. (2014) 'Oxidative Stress and Psychological Disorders', *Current Neuropharmacology*. doi: 10.2174/1570159x11666131120230309.

78) Sanchis-Gomar, F., Perez-Quilis, C., Leischik, R. and Lucia, A. (2016) 'Epidemiology of coronary heart disease and acute coronary syndrome', *Annals of Translational Medicine*, 4(13), pp. 256–256. doi: 10.21037/atm.2016.06.33.

79) Scheuer, D. A. and Mifflin, S. W. (2017) 'Repeated intermittent stress exacerbates myocardial ischemia-reperfusion injury', *American Journal of Physiology-Regulatory, Integrative and*

Comparative Physiology. doi: 10.1152/ajpregu.1998.274.2.r470.

80) Schiavone, S., Jaquet, V., Trabace, L. and Krause, K. H. (2013) 'Severe life stress and oxidative stress in the brain: From animal models to human pathology', *Antioxidants and Redox Signaling*. doi: 10.1089/ars.2012.4720.

81) Schneiderman, N., Ironson, G. and Siegel, S. D. (2005) 'Stress and Health: Psychological, Behavioral, and Biological Determinants', *Annual Review of Clinical Psychology*. doi: 10.1146/annurev.clinpsy.1.102803.144141.

82) Schroeter, M., Jander, S., Witte, O. W. and Stoll, G. (1994) 'Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion', *Journal of Neuroimmunology*. doi: 10.1016/0165-5728(94)90010-8.

83) Sheikh, A.S., Yahya, S., Sheikh, N. S. and Sheikh, A. A. (2012) 'C-reactive protein as a predictor of adverse outcome in patients with acute coronary syndrome', *Heart Views*. doi: 10.4103/1995-705x.96660.

84) Sherwood, L. (2010) 'Human Physiology: From Cells to Systems', *Human Physiology*, 7th editio, p. 766. doi: 9781111577438.

85) Smith, S. M. and Vale, W. W. (2006) 'The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress', *Dialogues in Clinical Neuroscience*, pp. 383–395. doi: 10.1038/nrendo.2011.222.

86) Sorrells, S. F., Caso, J. R., Munhoz, C. D. and Sapolsky, R. M. (2009) 'The Stressed CNS: When Glucocorticoids Aggravate Inflammation', *Neuron*. doi: 10.1016/j.neuron.2009.09.032.

87) Sorrells, S. F. and Sapolsky, R. M. (2007) 'An inflammatory review of glucocorticoid actions in the CNS', *Brain, Behavior, and Immunity*. doi: 10.1016/j.bbi.2006.11.006.

88) Stephens, M. A. C. and Wand, G. (2012) 'Stress and the HPA Axis Role of Glucocorticoids in Alcohol Dependence', *Alcohol research : current reviews*. PMID: 23584113

89) Straub, R. H. and Cutolo, M. (2016) 'Glucocorticoids and chronic inflammation', *Rheumatology (United Kingdom)*, 55, pp. 116–1114. doi: 10.1093/rheumatology/kew348.

90) Strike, P. C. and Steptoe, A. (2003) 'Systematic review of mental stress-induced myocardial ischaemia', *European Heart Journal*. doi: 10.1016/S0195-668X(02)00615-2.

91) Szardien, S., Mollmann, H., Willmer, M., Akashi, Y. J., Hamm, C. W. and Nef, H. M. (2013) 'Mechanisms of Stress (Takotsubo) Cardiomyopathy', *Heart Failure Clinics*. doi: 10.1016/j.hfc.2012.12.012.

92) Timmers, L., Sluijter, J. P., van Keulen, J. K., Hoefer, I. E., Nederhoff, M. G., Gourmans, M. J., Doevendans, P. A., van Echteld, C. J., Joles, J. A., Quax, P. H., Piek, J. J., Pasterkamp, G. and de Klein, D. P. (2008) 'Toll-like receptor 4 mediates maladaptive left ventricular remodeling and impairs cardiac function after myocardial infarction', *Circulation Research*. doi: 10.1161/CIRCRESAHA.107.158220.

93) Tsigos, C. and Chrousos, G. P. (2002) 'Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress', in *Journal of Psychosomatic Research*. doi: 10.1016/S0022-3999(02)00429-4.

94) Unnerstall, J. R., Kopajtic, T. A. and Kuhar, M. J. (1984) 'Distribution of α2 agonist binding sites in the rat and human central nervous system: Analysis of some functional, anatomic correlates of the pharmacologic effects of clonidine and related adrenergic agents', *Brain Research Reviews*. doi: 10.1016/0165-0173(84)90030-4.

95) Vakeva, A. P., Agah, A., Rollins, S. A., Matis, L. A., Li, L. and Stahl, G. L. (1998) 'Myocardial infarction and apoptosis after myocardial ischemia and reperfusion: Role of the terminal complement components and inhibition by anti-C5 therapy', *Circulation*. doi: 10.1161/01.CIR.97.22.2259.

96) Vazquez, S., Garner, B., Sheil, M. M. and Truscott, R. J. (2000) 'Characterisation of the major autoxidation products of 3- hydroxykynurenine under physiological conditions', *Free Radical Research*. doi: 10.1080/10715760000300021.

97) Vinten-Johansen, J. (2004) 'Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury', *Cardiovascular Research*. doi: 10.1016/j.cardiores.2003.10.011.

98) Vishwakarma, V. K., Upadhyay, P. K., Gupta, J. K. and Yadav, H. N. (2017) 'Pathophysiologic role of ischemia reperfusion injury: A review', *Journal of Indian College of Cardiology*. doi: 10.1016/j.jicc.2017.06.017.

99) Wada, K., Montalto, M. C. and Stahl, G. L. (2001) 'Inhibition of complement C5 reduces local and remote organ injury after intestinal ischemia/reperfusion in the rat', *Gastroenterology*. doi: 10.1053/gast.2001.20873.

100) Wettinger, S. B., Doggen, C. J., Spek, C. A., Rosendaal, F. R. and Reitsma, P. A. (2005) 'High throughput mRNA profiling highlights associations between myocardial infarction and aberrant expression of inflammatory molecules in blood cells', *Blood*. doi: 10.1182/blood-2004-08-3283.

101) WHO (2016) *Cardiovascular diseases (CVDs) fact sheets*, *Who*. Available at: https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).

102) Won, E. and Kim, Y.-K. (2016) 'Stress, the Autonomic Nervous System, and the Immune-kynurenine Pathway in the Etiology of Depression', *Current Neuropharmacology*. doi: 10.2174/1570159x14666151208113006.

103) Yang, L., Zhao, Y., Wangm Y., Liu, L., Zhang, X., Li, B. and Cui, R. (2015) 'The Effects of Psychological Stress on Depression', *Current Neuropharmacology*. doi: 10.2174/1570159x13041508311505O7.

104) Yang, N., Ray, D. W. and Matthews, L. C. (2012) 'Current concepts in glucocorticoid resistance', *Steroids*. doi: 10.1016/j.steroids.2012.05.007.

105) Yang, Z., Day, Y. J., Toufektsian, M. C., Xu, Y., Ramos, S., Marshall, M. A., French, B. A. and Linden, J. (2006) 'Myocardial infarct-sparing effect of adenosine A2A receptor activation is due to its action on CD4+ T lymphocytes', *Circulation*. doi: 10.1161/CIRCULATIONAHA.106.649244.

106) Yellon, D. M. and Hausenloy, D. J. (2007) 'Myocardial Reperfusion Injury', *New England Journal of Medicine*, 357(23), pp. 2408–2410. doi: 10.1056/NEJMc072913.

107) Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., Budaj, A.,

Pais, P., Varigos, J. and Lisheng, L. (2004) 'Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case control study', *The Lancet*, 364(9438), pp. 937–952. doi: 10.1016/S0140-6736(04)17018-9.

108) Zafir, A. and Banu, N. (2009) 'Induction of oxidative stress by restraint stress and corticosterone treatments in rats', *Indian Journal of Biochemistry and Biophysics*, 46(1), pp. 53–58.

109) Ziegler, M. G. (2012) 'Psychological Stress and the Autonomic Nervous System', in *Primer on the Autonomic Nervous System*. doi: 10.1016/B978-0-12-386525-0.00061-5.

110) Zuidema, M. Y. (2010) 'Ischemia/reperfusion injury: The role of immune cells', *World Journal of Cardiology*. doi: 10.4330/wjc.v2.i10.325.

111) Zupancic, M. L. (2009) 'Acute Psychological Stress as a Precipitant of Acute Coronary Syndromes in Patients With Undiagnosed Ischemic Heart Disease', *The Primary Care Companion to The Journal of Clinical Psychiatry*. doi: 10.4088/pcc.08r00623.

112) Zwacka, R. M., Zhang, Y., Halldorson, J., Schlossberg, H., Dudus, L. and Engelhardt, J. F. (1997) 'CD4+ T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver', *Journal of Clinical Investigation*. doi: 10.1172/JCI119533.

Chapter 2

An assessment of ischemia-reperfusion injury in rats exposed to chronic psychological stress

ABSTRACT

The stress response is a coordinated physiological response to external or internal stressors that aims to ultimately reestablish homeostasis. However, chronic activation of this system can lead to the disruption of cellular and systemic processes that can result in the development of neurological or psychosomatic diseases. Of note, psychological stress is recognized as a modifiable risk factor in the development of myocardial infarction, although underlying mechanisms driving this process remain unclear. This study therefore aimed to establish a model of chronic stress to determine whether it renders the heart more susceptible to damage following ischemia-reperfusion. Male Wistar rats were divided equally into a control and stress group (n = 12). Rats in the stress group were housed individually while control rats were housed two per cage. The stress group were subjected to the unpredictable chronic mild stress model, i.e. exposed to one random stressor six days a week over an eight-week period. At the end of this period, rat hearts were excised and employed for ex vivo heart perfusions while spleen tissue and plasma were used for oxidative stress and inflammatory tests, respectively. Our data reveal that circulating adrenocorticotropic hormone levels were lower within the stress group versus controls ($p < 0.05$), while corticosterone concentrations remained unchanged. Various markers for oxidative stress and inflammations showed no significant differences between the experimental groups. Following ischemia-reperfusion, the stress group displayed an increased infarct size compared to the control group ($p < 0.05$). These data show that the stress group experienced a degree of chronic stress that potentially renders the rat heart more susceptible to damage following ischemia-reperfusion.

OPSOMMING

Die stress reaksie is ‘n gekoordineerde fisiologiese reaksie op interne en eksterne stressors, met die eind doel om homestase te herstel. Alhoewel, kroniese aktivering van die sisteem kan lei tot die ontwrigting van sellulere en sistemiese prosesse wat kan lei tot die ontwikkeling van neurologiese of psigosomatiese siektes. Opmerklik word sielkundige stres erken as 'n veranderlike risikofaktor in die ontwikkeling van miokardiale infarksie, alhoewel die meganisme wat betrokke is nie duidelik is nie. Miokardiale infarksie is skade aan die hart as gevolg van ‘n vermindering van bloedvloei na miokardiale weefsel. Alhoewel, dit is egter bekend dat herstel van die bloedvloei self verdere skade aan die miokardium aandoen. Dit staan bekend as dodelike reperfusiebesering. Kroniese stress en iskemie-reperfusiebesering is beide geassosieer met 'n prominante inflammatoriese reaksie en die induksie van oksidatiewe stres. Die doel van hierdie studie was om 'n model van kroniese stres op te stel om te bepaal of kroniese stres die hart meer vatbaar maak vir skade na iskemie-reperfusie. Mannetjie Wistar-rotte is eweredig in 'n kontrolegroep en stresgroep verdeel (n = 12). Rotte in die stresgroep is afsonderlik gehuisves, terwyl kontrolerotte twee per hok gehuisves was. Die stresgroep is blootgestel aan die onvoorspelbare kroniese ligte stress model. Hulle is ses dae per week vir agt weke aan een willekeurige stressor blootgestel. Nadat rotte geëuthaniseer is, is die harte uitgesny en vir *ex vivo* hartvperfusies gebruik, terwyl miltweefsel en plasma vir biochemiese ontledings gebruik is. Miltweefsel is gebruik vir oksidatiewe stress ontledings, terwyl plasma gebruik was inflammatoriese en stresverwante merkers. Adrenokortikotropiese hormoon was laer in die stres groep ($p < 0.05$), terwyl corticosteron onveranderd gebly het. Oksidatiewe stress en inflammatoriese toetse het geen beduidende verskille tussen groepe getoon nie. Na iskemie-reperfusie het die stres groep ‘n groter infark gehad as die kontrole groep ($p < 0.05$). Ter afsluiting, daar was tekens dat die stress groep wel ‘n graad van kroniese stress ervaar het. Ons resultate dui dat kroniese stress die hart meer vatbaar maak vir skade na iskemia-reperfusie. Alhoewel, die meganisme verantwoordelik vir die skade moet meer ondersoek word, aangesien inflammatories en oksidatiewe stress toetse onoortuigend was.

1. Introduction

Hans Selye who is known as the modern-day ‘’father of stress’’ defined stress as the body’s non-specific response to any demand (Goldstein and Kopin, 2007) that can either be of an intrinsic or extrinsic nature (Nicolaides *et al.*, 2015). The body relies on two mechanisms to try and cope with a stressor: i) the HPA-axis and ii) the autonomic nervous system (Stratakis and Chrousos, 1995; Nicolaides *et al.*, 2015; Agorastos *et al.*, 2018). Activation of the HPA-axis ultimately results in secretion of cortisol (or corticosterone in rodents) the main glucocorticoid involved in the stress response (Nicolaides *et al.*, 2015; de Kloet *et al.*, 2016). When cortisol levels increase, it elicits a negative feedback action through binding to GR and MR (Juruena, 2014) to help control circulating cortisol levels and maintaining homeostasis. When bound to the GR, cortisol also elicits anti-inflammatory effects through suppressing the expression of NFκB within the nucleus (Cruz-Topete and Cidlowski, 2014).

Even though such an adaptive response is a coordinated physiological one that maintains homeostasis, chronic activation of this system can lead to the disruption of cellular and systemic processes resulting in dysfunction of both the nervous system and peripheral organ systems (Duric *et al.*, 2016; Agorastos *et al.*, 2018). Chronic activation of the HPA-axis is known to lead to GR resistance, impaired negative feedback and ultimately cortisol depletion (Hannibal and Bishop, 2014). With GR resistance, cortisol fails to bind to the GR and ultimately its anti-inflammatory effects and negative feedback actions are diminished (Cohen *et al.*, 2012). Moreover, chronic stress has been linked with increased levels of oxidative stress (Aschbacher *et al.*, 2013). Thus chronic stress leads to the dysfunction of the systems that regulate the stress response, ultimately resulting in the development of neurological and psychosomatic diseases (Duric *et al.*, 2016). Of note, psychological stress has been identified as a modifiable risk factor for the development of myocardial infarction (Yusuf *et al.*, 2004).

Myocardial infarction occurs as a result of decreased myocardial blood supply (Hoffman *et al.*, 2004; Lu *et al.*, 2015). However, restoring blood flow to the previously ischemic area can itself be a double edged sword, i.e. lethal reperfusion injury can exacerbate tissue damage and death following reoxygenation of the myocardium (Vinten-Johansen, 2004; González-Montero *et al.*, 2018). As a robust inflammatory response and the induction of oxidative stress are key factors that mediate ischemia-reperfusion injury (Vinten-Johansen, 2004; Kalogeris *et al.*, 2014; González-Montero *et al.*, 2018), any risk factor that enhances the inflammatory or oxidative stress response (e.g. chronic psychological stress) could potentially exacerbate reperfusion injury. However, despite such implied links, the mechanisms driving stress-related myocardial injury in the setting of ischemia-reperfusion remain relatively poorly understood.

The aims of this study were therefore to establish a rat model of chronic stress to evaluate inflammatory and oxidative stress markers, and to ultimately assess the effects of chronic stress on *ex vivo* ischemia-reperfusion. Here we hypothesized that rats experiencing chronic stress will develop a pro-inflammatory and pro-oxidative phenotype thereby rendering the heart more susceptible to damage following ischemia and reperfusion.

2. Materials and methods

2.1. Animals

Ethical approval was received prior to the start of this study from the animal ethics committee of the Stellenbosch University (South Africa) (ACU-2018-6311) (Appendix A). The animals used in this study were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Academy of Science (NIH publication No. 85-23, revised 1996). Eight-week-old male Wistar rats (200- 250g) were divided into three groups; control (n = 12), stress (n = 12) and negative control (n = 8). The stress rats were housed individually, while the control group were housed two per cage. This is due to social isolation potentially acting as a stressor to the control group and interfering with the validity of the study. The two groups were also housed in different rooms. Negative control rats were not handled at all and were only used for perfusion and biochemical analyses. All rats were subjected to a normal light-dark cycle (i.e. lights on from 9 am to 9 pm). Rats had access to *ad libitum* food and water. Room temperature was maintained at 22°C ± 1°C. The animals had one week to acclimatize to the new environment before the start of the study.

2.2. Unpredictable chronic mild stress (UCMS) model

The unpredictable chronic mild stress (UCMS) model was used for the duration of the study (Frisbee *et al.*, 2015). The stressed rats were subjected to one random mild stressor during the light phase six days a week (Figure 2.1). These stressors ranged from four to eight hours, depending on the stressor and included no bedding, damp bedding, predator smells and a tilted cage. A full description of the stressors is provided in Table 2.1. Stressors were randomized each week to minimize habituation and maintain its unpredictability. On the seventh day of the week body weight and food consumption were measured (Figure 2.1). Food pellets were weighed at the start and end of each week to determine their weekly food consumption. The animals were subjected to a blood draw during the seventh week – to

be discussed later. After eight weeks, the rats were euthanized, and blood and tissues were collected, snap frozen and stored at -80°C for further analyses (Figure 2.2).

Table 2.1: Description of the stressors used in the study.

Stressor	Description	Duration (hours)
No bedding	All bedding is removed and rats are placed into empty cages.	**4**
Damp bedding	Water is added to bedding. Pooling of water was avoided.	**8**
Cage tilt	Cages are tilted at an angle of ~40°. Bedding is also removed.	**8**
Predator	Concentrated predator urine (The Pee Mart, Vassalboro ME) is sprayed on the bedding of each cage.	**4**
Social stress	Rats are placed in the cage of another rat.	**4**
Light/dark	Lights are switched on and off every 30 minutes.	**8**

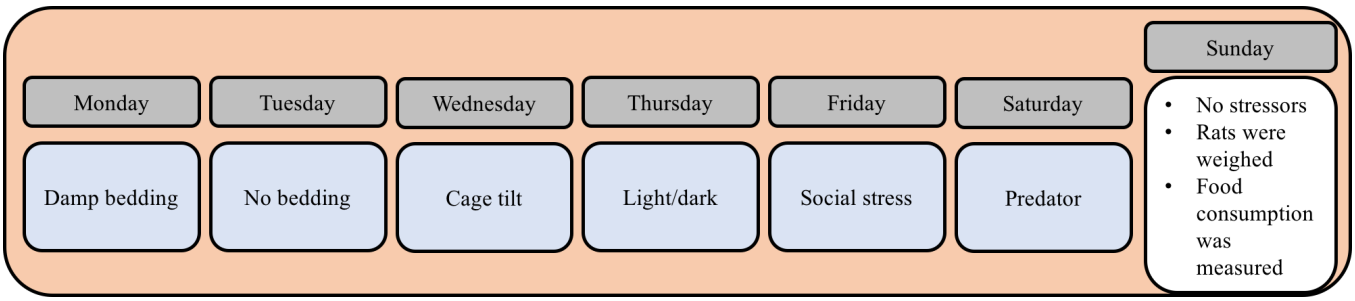


Figure 2.1: Weekly protocol. An example of a typical week for the stress group.

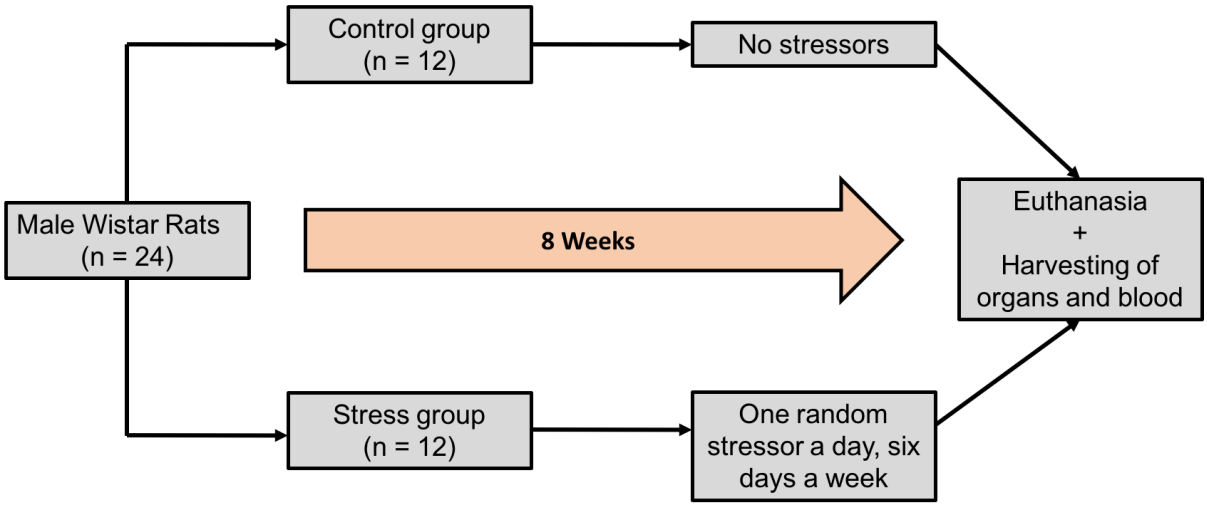


Figure 2.2: Experimental outline. Summary of the general experimental protocol. Blood draw took place during the 7th week of the protocol.

2.3. Pilot study

A pilot study was also conducted *before* the current study in order to establish the UCMS rat model in our laboratory. The aim was to confirm whether the rats developed a phenotype of chronic stress. This was a joint study that was completed by Lukas Olivier and Lucien Sher at Stellenbosch University. Here the data revealed that the stressors and experimental protocols adopted were not sufficient to induce adequate stress in the animals. All results obtained during pilot study added to supplementary data sheet (Appendix E). These results were, however, still useful as it allowed us to make alterations to our protocol to ensure the success of the current study (Table 2.2). Analyses included markers of lipid peroxidation, antioxidant capacity, oxidative stress status, inflammation and corticosterone.

Table 2.2: Changes made to the UCMS protocol following the pilot study.

Pilot study	Current study
Stressors during dark phase.	Stressors during light phase.
Control rats were housed individually.	Control rats were housed 2 per cage.
Control and stress rats were housed in same room.	Control and stress rats were housed in separate rooms.
Cage tilt, damp bedding and light/dark stressors lasted 4 hours each.	Cage tilt, damp bedding and light/dark stressors increased to 8 hours.
Cat sand and cat hair were used for predator stressor.	Concentrated bobcat urine was acquired for predator stressor.

2.4. Blood collection

Rats were anesthetized using 5% isoflurane gas (Piramel, Bethlehem PA) exactly one week before the end of the eight week protocol. Approximately 1 mL of blood was drawn from the right carotid artery. The blood was immediately transferred into ethylenediaminetetraacetic acid (EDTA) vacutainers (BD, Franklin Lakes NJ). The samples were centrifuged at 1, 000 x g for 15 minutes at 4°C (Boeco M240, Hamburg, Germany). Supernatant was transferred into a microfuge tube, snap frozen in liquid nitrogen and stored at -80°C for later analyses.

2.5. Euthanasia and tissue/blood collection

After the eight-week protocol, rats were injected with an overdose of sodium pentobarbital. The hearts were excised and used for *ex vivo* heart perfusions. Pooling blood in the chest cavity was collected using a 5 mL syringe and transferred to an EDTA tube. The EDTA tubes were subjected to centrifugation for 15 minutes at 1, 000 x g and 4°C. Plasma supernatant was stored in microfuge tubes and frozen at -

80°C. The brain, liver, kidneys, adrenals, pancreas, spleen and gonads were all collected in similar tubes and snap frozen in liquid nitrogen and then stored at -80°C for future analyses.

2.6. *Ex vivo* heart perfusions

Hearts were perfused retrogradely (Langendorff method) at a constant pressure (100cm H_2O). The heart was switched between Langendorff and working heart mode to assess functional capacity as well as induce ischemia and reperfusion (refer to Figure 2.3 for perfusion protocol) (Lochner, Genade and Moolman, 2003). Krebs-Henseleit bicarbonate buffer (119 mM NaCl, 25 mM $NaHCO_3$, 4.75 mM KCl, 1.2 mM KH_2SO_4, 0.6 mM $MgSO_4.7H_2O$, 0.6 mM Na_2SO_4, 1.25 mM $CaCl_2.H_2O$, D–(+)-glucose) (Appendix D) was used as the perfusate and was oxygenated and kept at a pH of 7.4 by gassing (95% O_2. 5% CO_2). The perfusion setup was coated with a water jacket to maintain an internal temperature of ~36.8°C.

To induce regional ischemia, the left anterior descending (LAD) coronary artery was tied off using a silk suture and the temperature of the heart was monitored and kept at ~ 36.8 °C. After ischemia the knot was undone, and the heart was reperfused for 10 minutes and given the chance to recover. Functional recovery was assessed by comparing function prior to and after ischemia and was expressed as a percentage (Appendix D).

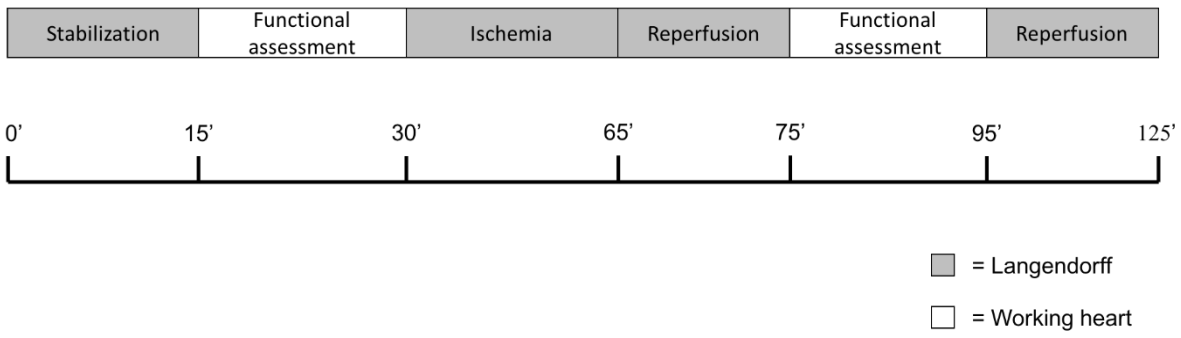


Figure 2.3: ***Ex vivo* perfusion protocol.** Time points and perfusion methods during the *ex vivo* heart perfusions**.**

2.6.1. Infarct size determination

After the perfusion protocol the suture was tightened at the same spot and a 0.5% Evans Blue solution was slowly injected through the aorta (Lochner, Genade and Moolman, 2003). The heart was frozen overnight at -10°C before being sectioned into five equally thick slices (~ 2mm). The heart was cut from the apex towards the knot (transverse). The slices were incubated in 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for ~ 7 minutes. The slices were then fixed in a 10% formaldehyde solution for approximately one hour to enhance the contrast between the viable tissue and the infarcted area. The viable tissue (V), area at risk (AR) and infarct zone (I) was sketched onto transparent paper (Figure 2.4). ImageJ (National Institutes of Health, USA) was used to determine the area of these sections and to calculate the infarct size. Infarct size and area at risk were calculated as follows:

$$Infarct\ size = \frac{I}{(AR + I)} \times 100 \qquad Area\ at\ risk\ size = \frac{(AR + I)}{Total\ area} \times 100$$

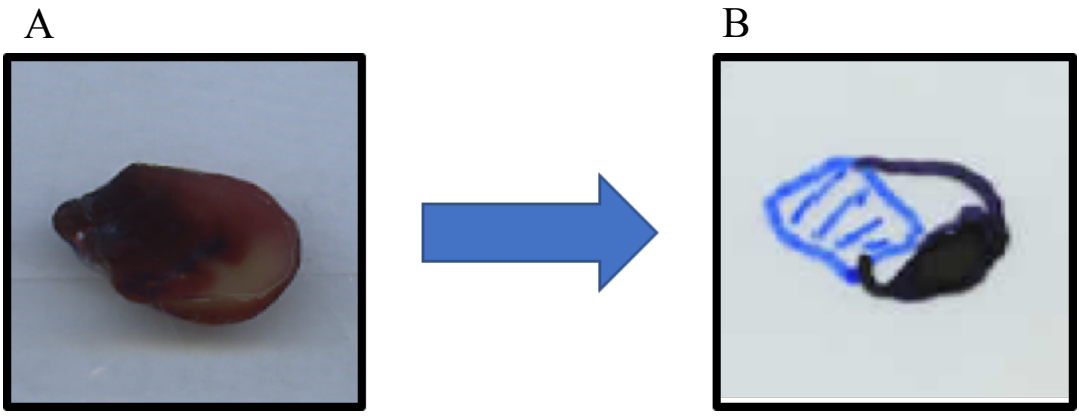


Figure 2.4: Example of how viable tissue (V), area at risk (AR) and infarct zone (I) was represented in a drawing (B). The blue area represents viable tissue, the black is the infarct, and the rest is the area at risk (AR).

2.7. Plasma analyses

ELISA kits for ACTH (E-EL-R0048), corticosterone (E-EL-R0269) and hs-CRP (E-EL-R0506) were acquired from Elabscience (Elabscience Biotechnology Inc, Houston TX). The ACTH and corticosterone kits used the competitive-ELISA principle, while the hs-CRP kit used the sandwich-ELISA principle (Appendix B). Plasma samples that were collected after the blood draw during the 7th week were used for the ACTH and corticosterone ELISA kits. Plasma samples collected following euthanasia were used for the hs-CRP ELISA. Plates were read at a wavelength of 490nm on the EZ Read 400 Microplate reader (Biochrom, Holliston MA) and results were calculated from the standard curve.

2.8. Tissue analyses

As there were no heart tissues available for biochemical analyses after perfusions, we decided to focus on the spleen. The spleen is an integral part of the lymphatic system and plays a role in the production of lymphocytes (Mitchell, 1973). Chronic inflammation can consequently induce oxidative stress and attenuate anti-oxidant capacity (Khansari, Shakiba and Mahmoudi, 2009). Spleen samples were prepared in a 1x PBS solution (Appendix C) at a 1:10 ratio for SOD and thiobarbituric acid reactive substances (TBARS). The TBARS assay measures natural byproducts of lipid peroxidation, like malondialdehyde (MDA). TBARS was read at a wavelength of 532 nm in the Multiskan® Spectrum microplate spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) (Appendix C). The SOD assay measures the auto-oxidation of 6-hydroxydopamine (6-HD) and it's activity was assessed over a period of five minutes on the Multiskan® Spectrum microplate spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) (Appendix C). Figure 2.5 summarizes all the analyses during and after sample collection.

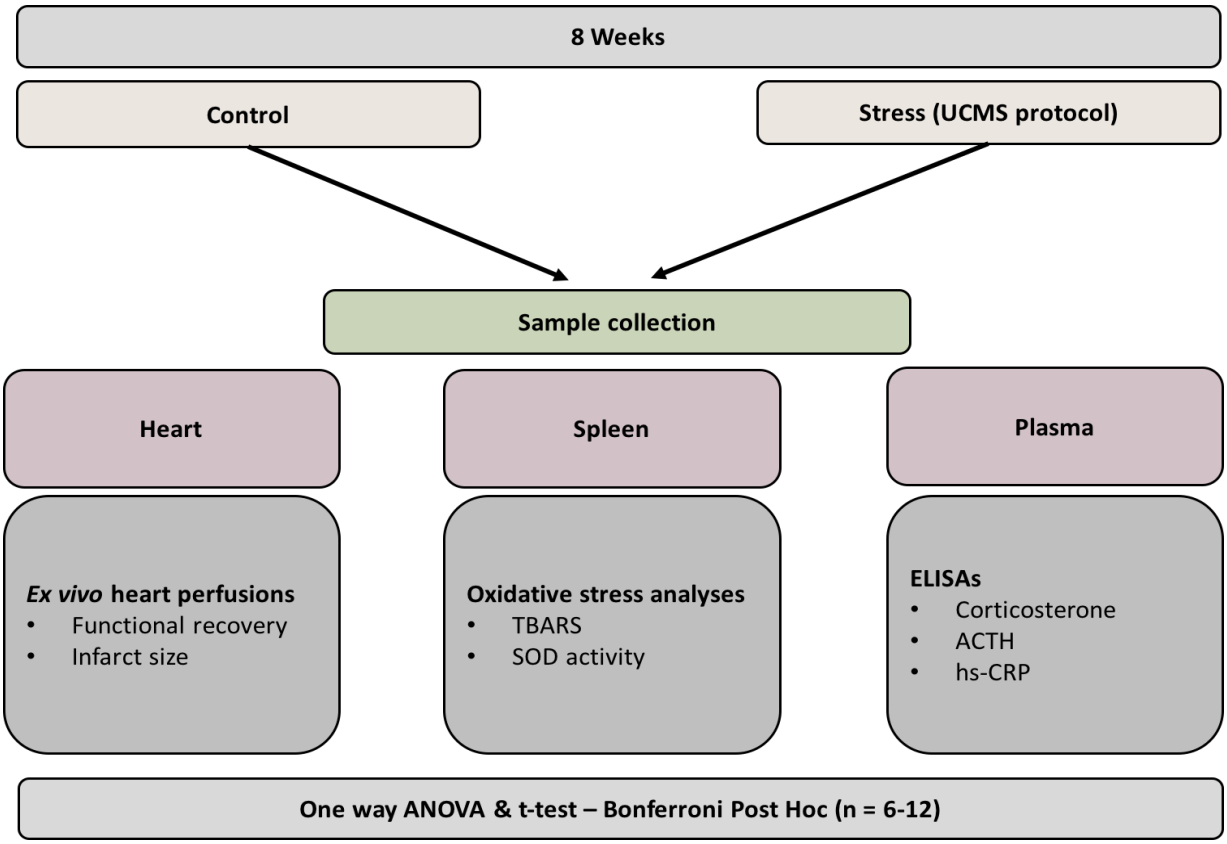


Figure 2.5: Experimental procedure. Schematic illustration and summary of the experimental procedure that was followed after sample collection. ACTH: adrenocorticotropic hormone, hs-CRP: high sensitivity C-reactive protein, SOD: superoxide dismutase, TBARS: thiobarbituric acid reactive substances, UCMS: unpredictable chronic mild stress.

2.9. Statistical analyses

Graphpad Prism 8 (Graphpad Software Inc, San Diego, CA) was used for all statistical analyses and data was analyzed with the assistance of Prof. Martin Kidd, a statistician based at the Centre for Statistical Consultation at Stellenbosch University. All data was tested for normality and the presence of any outliers. The Students t-test was used to compare the control and stress group. One-way ANOVA was used to compare control, stress and negative control group, with a Bonferroni *post hoc* test. Differences were considered significant if the p-value was less than 0.05. All data represented as mean ± SEM.

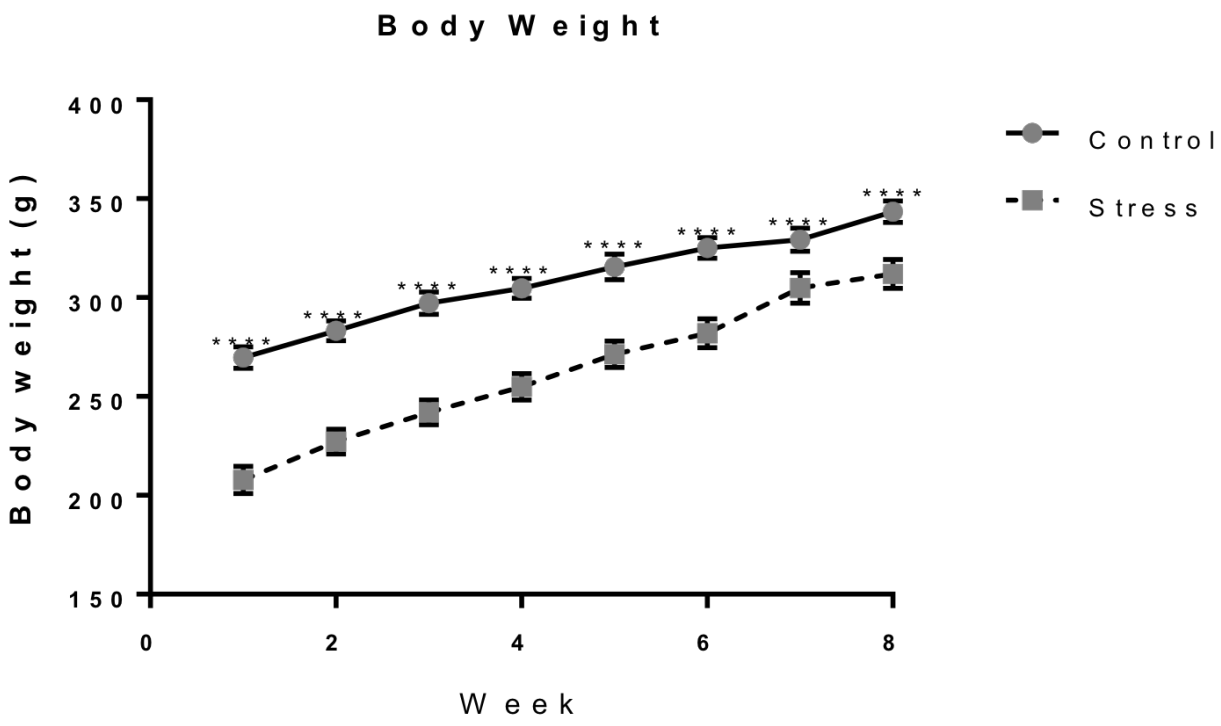
3. Results

As this was part of a joint study and plasma samples were shared, corticosterone and ACTH tests were done with a sample size of (n = 6). However, in combining the results with data acquired by Lucien Sher, we achieved a joint sample size of (n = 12). Body weight and food consumption data are also shared and accredited to both myself (Lukas Olivier) and Lucien Sher. All heart and spleen tissues were used by myself for *ex vivo* heart perfusions and oxidative stress analyses respectively.

3.1. Body weight and food consumption

Body weight was taken once a week for the duration of the eight week study. Here the control group weighed significantly more than the stress group at the start of the experiment ($p < 0.0001$) (Figure 3.1A). When the data are expressed as percentage growth this shows that the stress group gained significantly more weight throughout the eight week protocol (Figure 3.1B). Although the stress group gained more weight over the 8-week duration of the experiment ($p < 0.0001$) (Figure 3.1B), there was still a weight difference of 9.63 ± 2.81% at the end of the experiment, compared to the 25.93 ± 3.7% seen during week 1. During the first week the control rats consumed significantly more food than the stress group ($p < 0.05$), however the stress group consumed more food during the fifth ($p < 0.001$), sixth ($p < 0.001$) and eighth ($p < 0.05$) weeks (Figure 3.2). As the variation in rat weights at the end of the experiment may impact on our data analyses, correlation tests were completed (in collaboration with Prof. Martin Kidd of the Centre for Statistical Consultation at Stellenbosch University) to assess the impact of the body weight difference on other analyses performed. Such an analysis revealed that the difference in body weight was not a confounding factor and did not have any effect on any of our biochemical or perfusion analyses.

A



B

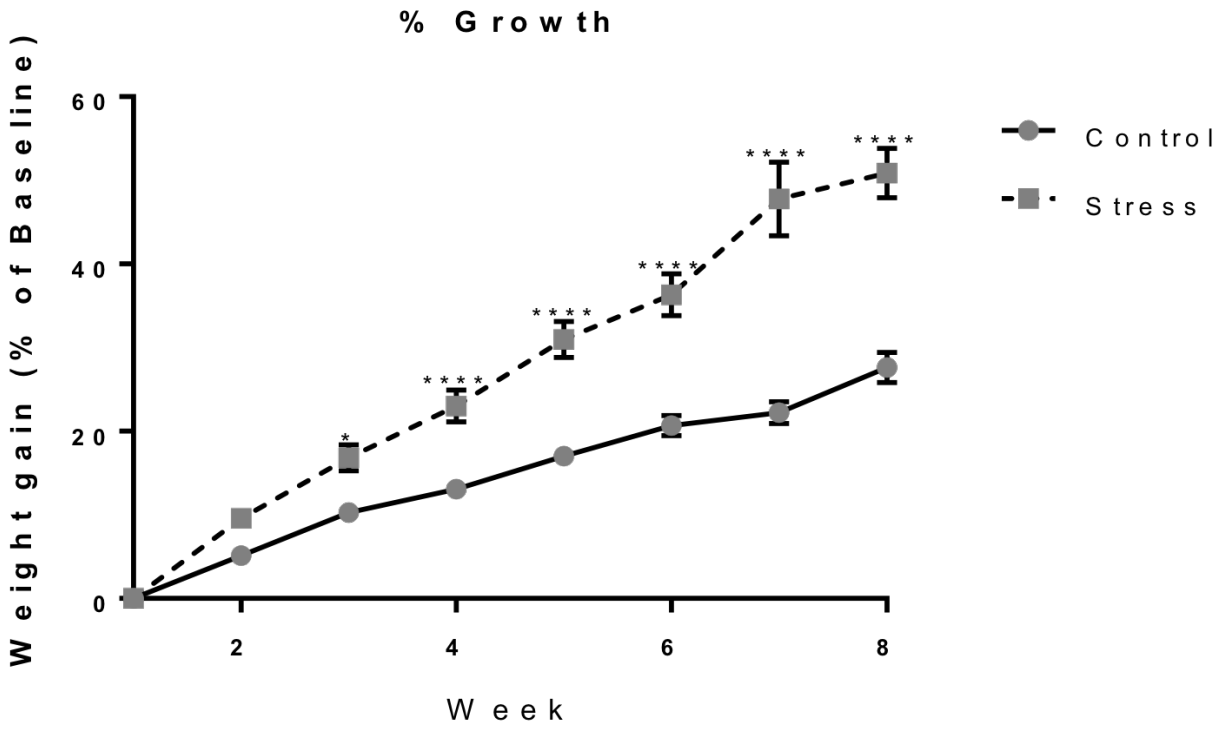


Figure 3.1: **Body weight (A) and percentage growth (B) results over the eight week period (Sher and Olivier, unpublished data).** Data presented as mean ± SEM; statistical analyses: repeated measures, two-way ANOVA, Bonferroni post hoc; *p<0.05, ****p<0.0001; n = 12.

Figure 3.2: **Food consumption over the eight week protocol (Sher and Olivier, unpublished data).** Data presented as mean ± SEM; statistical analyses: repeated measures, two-way ANOVA, Bonferroni post hoc; *p<0.05, ***p<0.001; n = 12.

3.2. *Ex vivo* heart perfusions

3.2.1. Functional parameters

Throughout the 125-minute perfusion protocol heart rate and coronary flow were assessed at various time points. Our findings show that there were no statistically significant differences between the groups

(Table 3.1). As expected our results confirm that the coronary flow was markedly attenuated during ischemia ($p < 0.0001$).

Table 3.1: **Heart rate and coronary flow before and after ischemia was induced (during Langendorff).** Data presented as mean ± SEM; statistical analyses: one-way ANOVA, Bonferroni post hoc; * $p < 0.0001$ for B vs. A & C. n = 10-11. BPM – beats per minute.

	Pre-ischemia (A)		**During ischemia (B)**	**Post-ischemia (C)**	
	Heart rate (BPM)	**Coronary flow (mL/min)**	**Coronary flow (mL/min)**	**Heart rate (BPM)**	**Coronary flow (mL/min)**
Control	271.7 ± 12.5	9.4 ± 0.5	5.2 ± 0.4 *	274.9 ± 7.2	8.6 ± 0.4
Stress	257.8 ± 19.8	9.3 ± 0.4	4.7 ± 0.3 *	262.8 ± 13.9	8.8 ± 0.4

3.2.2. Functional recovery

Functional recovery indicates how well the heart's functionality recovered following ischemia and reperfusion. The results are expressed as a percentage recovery of functionality following ischemia-reperfusion. Our data show no significant differences for functional recovery between the groups (Figure 3.3).

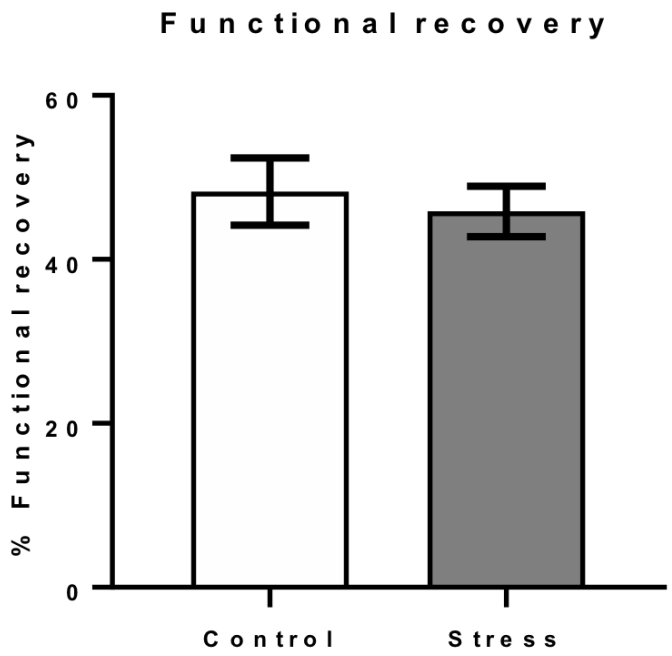


Figure 3.3: **Percentage recovery of functional ability after ischemia and reperfusion.** Data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 10-11.

3.2.3. Infarct size and area at risk

The stress group displayed an increased infarct size compared to the control group ($p < 0.05$) (Figure 3.4A). There were also no significant differences for the area at risk (Figure 3.4B), confirming that the LAD coronary arteries were identically ligated in both groups.

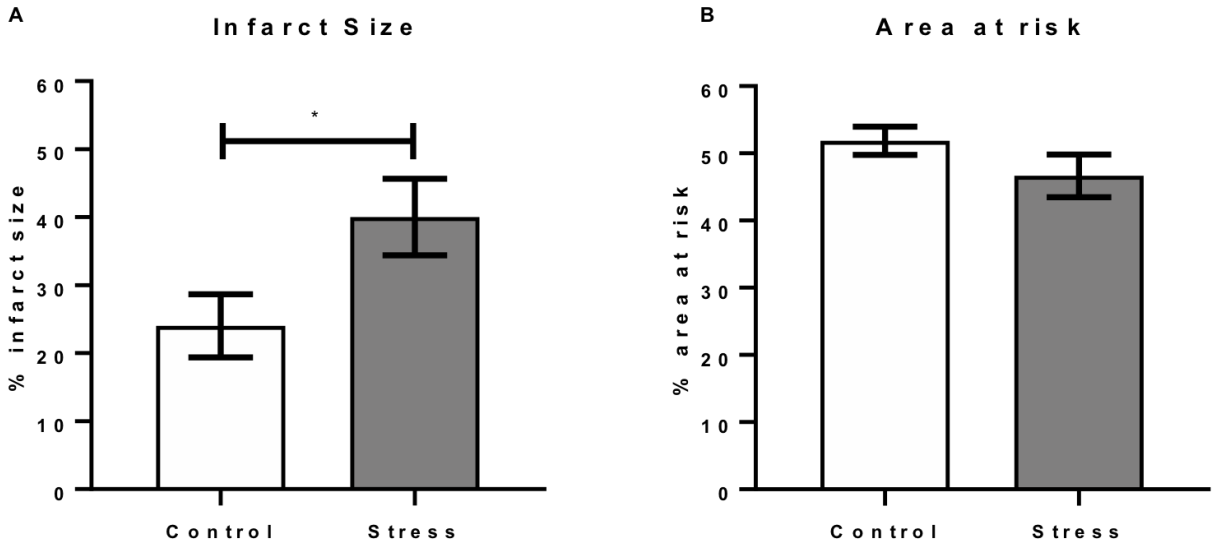


Figure 3.4: Size of infarct (A) and area at risk (B) displayed as a percentage. Data displayed as mean ± SEM; statistical analyses: unpaired t-test, Bonferroni post hoc; *p<0.05; n = 12.

3.3. Biochemical analyses

3.3.1. Stress related markers

Plasma corticosterone showed no differences between groups (Figure 3.5A). A similar pattern was observed with the combined data (for the larger study, in collaboration with Lucien Sher) (Figure 3.5B). However, our findings show lower ACTH levels in the stress group (Figure 3.6A) and this became significant when presented as combined data (Figure 3.6B).

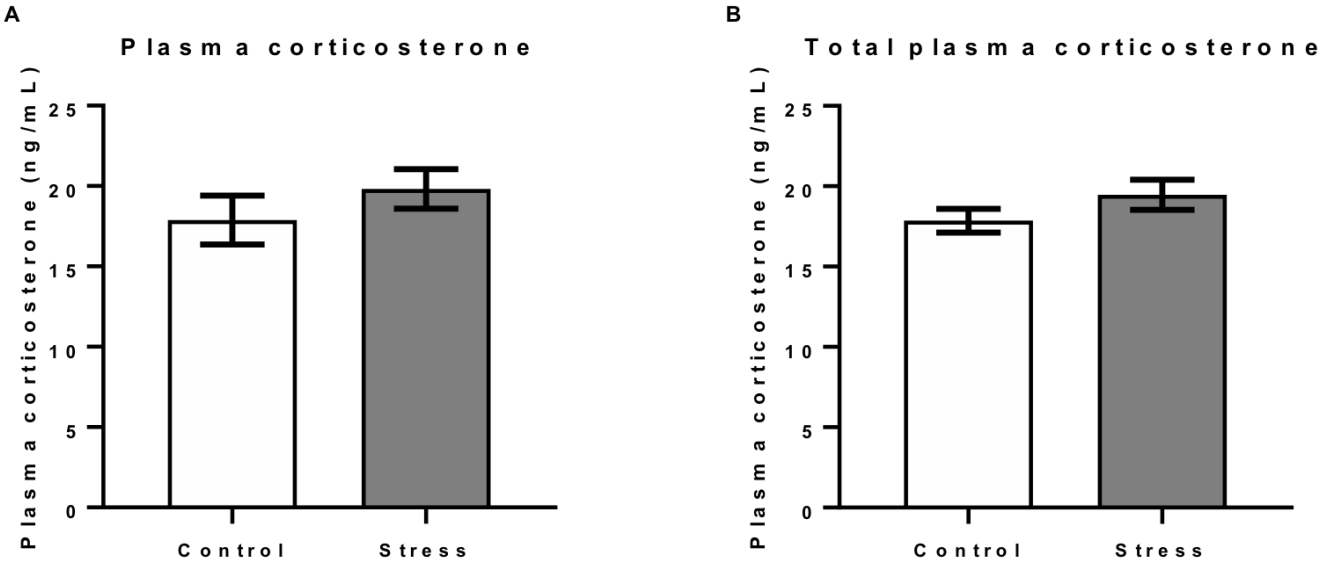


Figure 3.5: **Plasma corticosterone results of this study (A) (n = 6) and plasma corticosterone of joint study (Sher and Olivier, unpublished data) (B) (n = 12).** Data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 12.

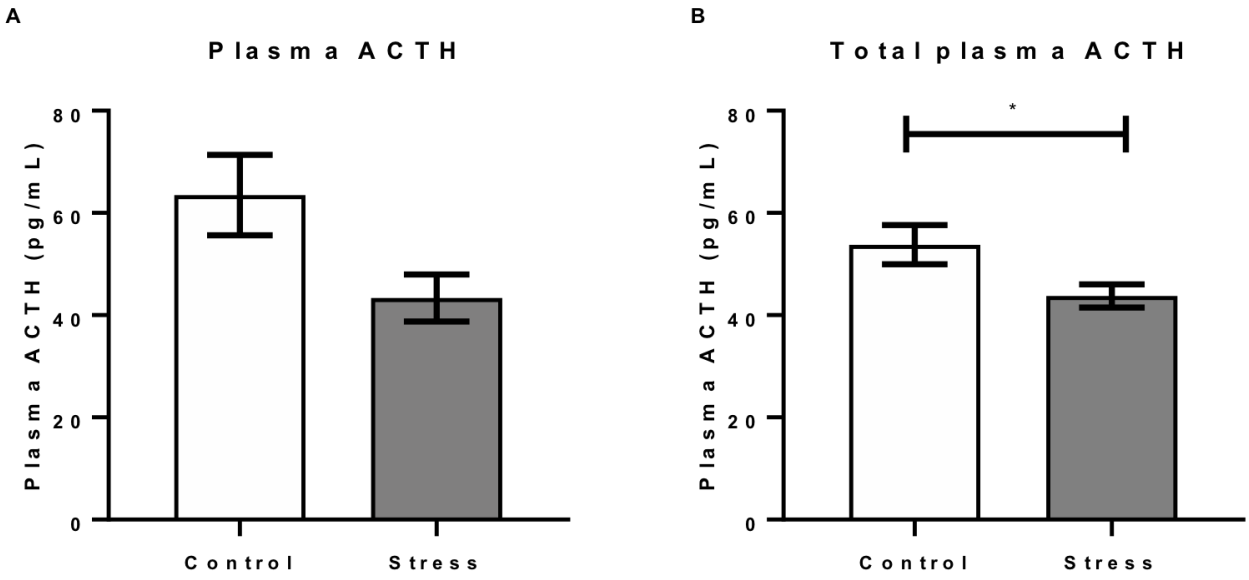


Figure 3.6: **Plasma ACTH results of this study (A) (n = 6) and plasma ACTH of joint study (Sher and Olivier, unpublished data) (B) (n =12).** Data presented as mean ± SEM; statistical analyses: unpaired t-test, Bonferroni post hoc; *p<0.05. ACTH: adrenocorticotropic hormone

3.3.2. Oxidative stress

As no heart tissue was available for biochemical analyses, spleen tissue was used to determine the oxidative stress activity following the stress protocol. However, SOD activity and MDA concentration in spleen tissue were similar in both groups (Figure 3.7).

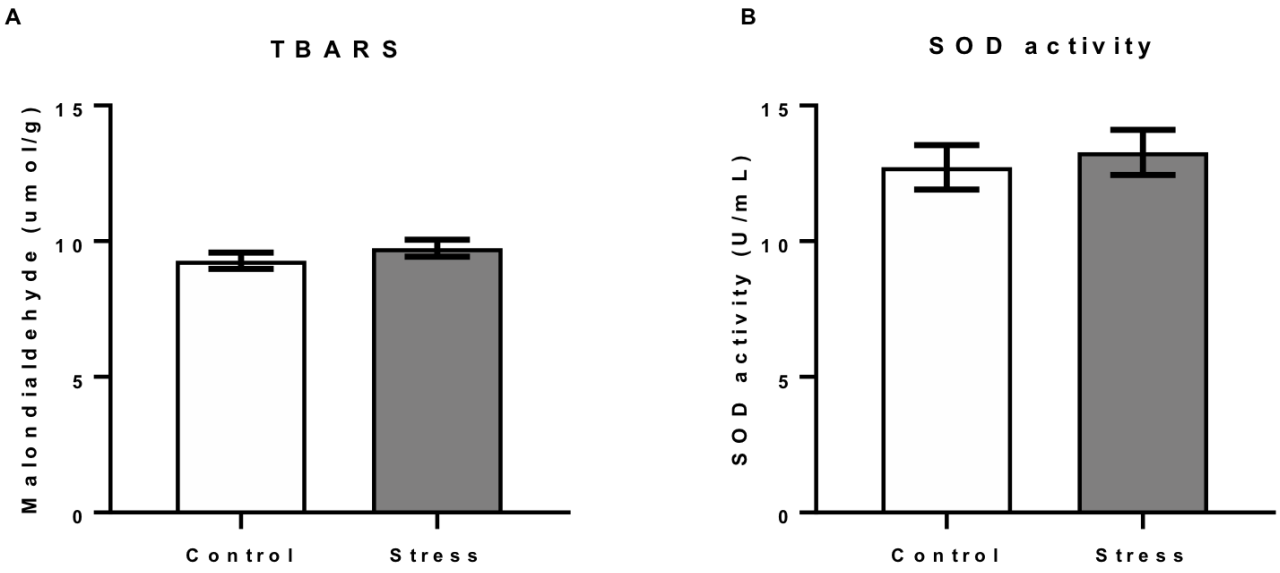


Figure 3.7: **Oxidative stress analyses. Malondialdehyde concentration (A) and superoxide dismutase activity (B) in spleen tissue**. Data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 12. SOD: superoxide dismutase

3.3.3. Systemic inflammation

CRP was measured within the plasma as it's a good marker for indicating any cardiovascular complications or stress related complications (Sheikh *et al.*, 2012). However, plasma analyses of hs-CRP showed no significant differences between groups (Figure 3.8).

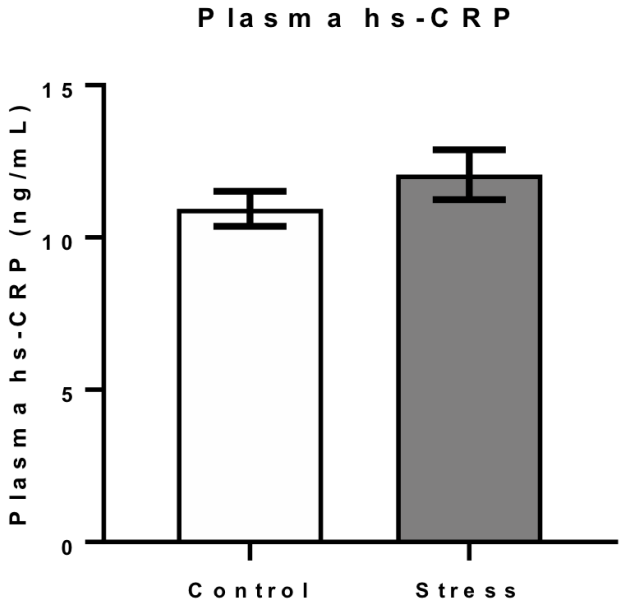


Figure 3.8: **Plasma concentration of hs-CRP** . Data displayed as mean ± SEM; statistical analyses: unpaired t-test; n = 12. CRP: C-reactive protein

4. Discussion

As discussed, chronic stress is increasingly being recognized as a risk factor in the development of cardiovascular disease (Yusuf *et al.*, 2004). As the underlying mechanisms driving this process remain unclear, this study aimed to establish a model of chronic stress with the aim to assess its impact in response to *ex vivo* ischemia-reperfusion. The main findings of this study include: i) the establishment of the UCMS model, and ii) increased infarct sizes in the stress group following ischemia-reperfusion.

4.1. The UCMS model

The UCMS model was chosen for this project as it is one of the most translationally relevant chronic stress models for studying the pathology of depression (Frisbee *et al.*, 2015). The UCMS model provides the opportunity for greater insight into molecular, genetic and epigenetic factors that may contribute to the onset of depression, as well as identifying potential therapeutic targets (Frisbee *et al.*, 2015). This model is widely used as it reproduces clinical symptoms of depression observed in humans e.g. anhedonia and learned helplessness (Frisbee *et al.*, 2015). Studies do indicate that unpredictable forms of stressors are more likely to induce depressive symptoms than restraint stress (Zhu *et al.*, 2014). Furthermore, such outcomes can be reversed by the use of anti-depressants, emphasizing the similarity between human depressive symptoms and the UCMS model (Frisbee *et al.*, 2015).

The use of preclinical animal models offers a great translational tool in studying stress-related illnesses (e.g. depression) and potential links to comorbidities. Of note, depression is increasingly present in patients with coronary heart disease and is independently connected to cardiovascular morbidity and mortality (Lichtman *et al.*, 2009). Apart from contributing to coronary heart diseases risk (Carney and Freedland, 2017), depression may render the heart resistant to cardioprotective interventions (Headrick *et al.*, 2017).

Behavioral and molecular tests (e.g. corticosterone and ACTH) are useful tools whereby models of chronic stress can be validated. As we did not perform any behavioral tests (due to excessive workload), we relied solely on molecular analyses to validate the UCMS model. These results will be thoroughly discussed.

In addition to anhedonia, models of chronic stress are known to reduce the rate of weight gain in rodents (Willner, 2017). For the current study we found contrasting results as the control group weighed significantly more than the stress group (Figure 3.1A). During the first week of the experiment the control group consumed significantly more food than the stress group, while the stress group consumed significantly more food during the fifth, sixth and eighth weeks. This correlates well with the percentage growth as the stress group gained significantly more weight over the eight week period (Figure 3.1B). However, after completion of the experiment we discovered that the rats were age-matched at the start of the experiment but regrettably not weight-matched. This unfortunate oversight was only detected at the end of the experiment and meant we had to complete additional statistical analyses to determine whether body weight was indeed a confounding factor in our study. Here detailed correlation tests were completed with the assistance of Prof. Martin Kidd, a statistician based at the Centre for Statistical Consultation at University of Stellenbosch. Such analyses revealed that the only significant body weight effect that we identified was a correlation with the infarct size data – to be discussed later.

Following our eight week protocol corticosterone levels remained unchanged between groups. Contrasting results are found in other studies also employing the UCMS model as they found significantly higher corticosterone levels in the C57BL/6J inbred mouse strain (Van Boxelaere *et al.*, 2017) and Wistar Kyoto rats (Bernatova *et al.*, 2018). Here the latter study used decapitation as the method of termination following carbon dioxide anesthesia. This method could potentially minimize additional unnecessary stress on the rats compared to our method of euthanasia (sodium pentobarbital injection).

Surprisingly, circulating ACTH levels were significantly lower in the stress group. As discussed, our initial thoughts were that during the anesthesia process the control group experienced a degree of stress that ultimately enhanced their stress response, thus expected to increase ACTH and corticosterone levels. However, this was not the case when assessing the ACTH data. Therefore, we propose that a method reflecting corticosterone levels over months is needed as it should provide a better reflection of underlying physiology and pathophysiology. Here the measurement of corticosterone in hair samples is increasingly becoming a suitable method for determining corticosterone concentrations over extended periods of time (Sauvé *et al.*, 2007; Golbidi, Frisbee and Laher, 2015). In support, Kalra and colleagues found that maternal hair cortisol levels correlated positively and significantly with measures of perceived stress (Kalra *et al.*, 2007). This would eliminate any chance of anesthesia-mediated stress interfering with our analyses. Thus, one possibility is that our corticosterone and ACTH findings may – in part – be explained by the choice of anesthetics for our study.

Another possibility also exists, i.e. reported GR resistance following chronic stress. During GR resistance corticosterone fails to bind to the GR, thus diminishing its anti-inflammatory effects and negative feedback functions (Cohen *et al.*, 2012; Yang, Ray and Matthews, 2012). We propose that such a negative feedback loop may be operational in our model, although additional studies are required to confirm this. This then begs the question why cortisol is similar, but ACTH significantly reduced in the stress group. In the absence of GR resistance, corticosterone is still able to elicit its negative feedback function. Thus, ACTH levels could be reduced in the stress group to compensate for the chronically elevated corticosterone levels and ultimately decrease adrenal corticosterone secretions. This could indicate that despite the stress group experiencing a certain degree of stress, the chronic stress protocol here employed was not severe enough to induce GR resistance.

Our data did not reveal any significant differences for plasma CRP concentrations between the control and stress groups. A previous study on rabbits reported elevated plasma levels of CRP when using the UCMS model (Lu *et al.*, 2012). The lack of differences in CRP levels supports the notion that our eight-week UCMS protocol was not severe enough to elicit changes in GR expression as other studies show

that GR resistance is often accompanied by a pro-inflammatory state (Sorrells and Sapolsky, 2007; Hannibal and Bishop, 2014; Headrick *et al.*, 2017). As discussed, corticosterone is prevented from binding to GR in the cytoplasm in the case of GR resistance. Consequently, GR fails to translocate into the nucleus and bind to the GREs within the DNA (Padgett and Glaser, 2003; Barnes, 2015). Transcriptional activity of the downstream genes are subsequently altered (Sorrells and Sapolsky, 2007), i.e. the inability of GR to bind to GREs results in the failure to downregulate the transcriptional activity of NFκB and AP-1 and the anti-inflammatory properties of corticosterone are diminished (Sorrells and Sapolsky, 2007; Straub and Cutolo, 2016). Thus we suggest that future studies should focus on NFκB as a driver of a pro-inflammatory milieu (Clark, 2007).

As all hearts were used for *ex vivo* heart perfusions and infarct size determination there were no cardiac tissues available to assess oxidative stress. We therefore decided to do an assessment using spleen tissue as it is one of the largest secondary immune organs and plays an important role in disease development (e.g. myocardial infarction) (Swirski *et al.*, 2009; Jiang *et al.*, 2017). Of note, enhanced ROS generation by immune cells can lead to a state of oxidative stress (Chatterjee, 2016). However, there were no signs of lipid peroxidation or changes in SOD activity within the spleen tissue. By contrast, a study using a chronic social isolation stress model in rats found increased spleen MDA concentrations after 13 weeks (Gavrilović *et al.*, 2018). This adds support to the concept that our UCMS model represents a moderate phenotype. As there was no systemic inflammation in our model and as it is known to play a role in the induction of oxidative stress (Chatterjee, 2016), this could explain why there were no signs of lipid peroxidation or any changes in SOD activity in this case. Of note, additional oxidative stress analyses were conducted on liver and brain tissue by other researchers in our group (Essop laboratory, unpublished data). These results reveal increased oxidative stress in both tissues thus supporting a moderate stress phenotype in our UCMS model. We can also speculate that the UCMS model might be simulating signs of post-traumatic stress disorder (PTSD) in the rats. Here the unchanged corticosterone levels support the fact that with PTSD there is lowered HPA-axis activity (Kolassa *et al.*, 2007). Unresponsive HPA-axis also correlates well with our ACTH findings, although more studies are required to investigate such propositions.

4.2. Increased infarct size following ischemia-reperfusion

There are paradoxical reports about the effects of stress on the cardiovascular system. For example, acute stress is known to decrease myocardial sensitivity to ischemia-reperfusion injury (Eisenmann, Rorabaugh and Zoladz, 2016), while others found that chronic stress exacerbates ischemia-reperfusion injury (Table 1.1). Results from the current study support the majority of these findings as the infarct size of the stress group was significantly increased following ischemia-reperfusion protocol. Although it is established that chronic stress exacerbates ischemia-reperfusion injury, the potential mechanisms contributing to this process still remain elusive. We therefore hypothesized that potential mechanisms involved is likely to be of a pro-inflammatory and pro-oxidative nature within the heart and/or impaired corticosterone catabolism.

Cortisol is well-known to inhibit insulin and ultimately increase blood glucose levels (Al-sharefi, 2016). Chronic stress also induces insulin-resistance thereby contributing to a hyperglycemic state (Tsigos *et al.*, 2000; Kyrou and Tsigos, 2007). Both hyperglycemia and insulin-resistance are known to impair infarct tolerance and cardioprotective signaling (Hausenloy *et al.*, 2013). However, we found no significant differences in plasma corticosterone levels between groups. Interestingly, chronic stress induces long-term lowering of glucocorticoid catabolism (Yehuda and Seckl, 2011), suggesting that the actions of corticosterone in the stress group are prolonged compared to the control group despite plasma concentrations being similar. Thus, we propose that altered corticosterone catabolism could be a factor responsible for the aggravated injury. However, further studies are required to investigate this idea. As increased inflammation and oxidative stress are indeed major contributors to ischemia-reperfusion injury within the heart (Zuidema, 2010; Eltzschig and Eckle, 2011; Kalogeris *et al.*, 2014), it remains a possibility that chronic stress induced a pro-inflammatory and pro-oxidative state within the heart that aggravated the ischemia-reperfusion injury. Little is known about the mechanistic features of stress or depression aggravated infarction, with the majority of literature limited to expression of anti- and pro-apoptotic proteins (Wang *et al.*, 2013). Hence more studies need to done to assess the molecular effects

of chronic stress on myocardial inflammation and oxidative stress prior to and after ischemia-reperfusion in order to ascertain what exactly is causing the aggravated injury.

As rats were not weight matched and the difference between groups were significant, correlation tests were completed to assess if there were any correlations between these results and body weight at the time of euthanasia. As mentioned before, the only significant correlation identified was between infarct size and body weight. Thus the possibility exists that infarct size was not significantly altered by our stress protocol, but instead being representative of body weight differences. However, correlation does not always mean causation. As all the rats in the control group weighed significantly more than the stress group, it means that any other significant finding relating to the two experimental groups would indeed correlate either positively or negatively with body weight. Moreover, as infarct size was the only parameter that correlated with body weight, we speculate that the change in infarct size was not due to body weight but rather as a result of the chronic stress protocol. However, further studies are required to conclusively prove this notion.

Although infarct size results indicated promising results, there were no significant differences in functional recovery and other functional parameters between the groups following ischemia and reperfusion. The coronary flow was reduced significantly during ischemia (for both groups) as would be expected but increased again following reperfusion. Of note, a decrease in infarct size is not always associated with improved functional recovery, thus indicating that increased infarct size is not necessarily be associated with decreased functional recovery (Lochner, Genade and Moolman, 2003).

4.3. Limitations

As discussed, the fact the rats were not weight matched remains a limitation of this study. During this taxing study we were unable to complete any behavioral tests due to sheer workload, making it difficult to fully establish whether the rats were sufficiently stressed after the eight week protocol. Blood pressure analyses were done at the start, middle and end of the study to serve as another method of

confirming the onset of stress. However, technical difficulties with the equipment meant that we could not use these data due to significant variability and inaccuracies. Of note, this method included restraining the rats in a small Perspex tube for the duration of the analysis and hence exposed our control rats to a stressor that could potentially impact on our data.

5. Conclusion

Cardiovascular disease remains a major, global health problem contributing to ~ 31% of global yearly deaths (WHO, 2016). Apart from well known risk factors, chronic psychological stress is increasingly associated with the onset of myocardial infarctions (Yusuf *et al.*, 2004). Both chronic psychological stress and ischemia-reperfusion injury are associated with increased oxidative stress and inflammation. The aim of this study was to assess the pro-inflammatory and pro-oxidative effects of chronic stress, and whether it would render the heart more susceptible to ischemia-reperfusion injury. Following eight weeks of the UCMS protocol, the collective data suggest that the stress group did experience a degree of chronic stress together with increased infarct sizes compared to the control group. Even though there were no signs of oxidative stress or systemic inflammation, chronic stress still impaired the heart's infarct tolerance and potentially interfered with cardioprotective mechanisms. This study highlights the complex interplay between the brain and the heart and the importance to better understand the brain-heart axis. Such efforts should eventually help to lower the prevalence of cardiovascular-related complications that stem from chronic stress-related illnesses.

5.1. Future recommendations

For future studies it is important that behavioral tests be added (e.g. sucrose preference test, sucrose spray test) to better assess stress levels of stress in the model. This should assist in determining the efficacy of the protocol. In addition, measuring corticosterone and ACTH levels at different time points may also provide greater insight regarding the nature of the UCMS model, while a different method of

measuring corticosterone levels (e.g. hair corticosterone) should provide a ‘snapshot’ of levels over the past few months. An important addition could be to increase the sample size to assess myocardial inflammation and oxidative stress prior to and after ischemia reperfusion. This should provide greater insights regarding underlying mechanisms at play in the heart following eight weeks of the UCMS model. Lastly, assessing metabolic markers such as circulating insulin and glucose levels may lead to further insights regarding corticosterone effects in the stress group.

6. References

1) Agorastos, A., Pervanidou, P., Chrousos, G. & Kolaitis, G. (2018) 'Early life stress and trauma: developmental neuroendocrine aspects of prolonged stress system dysregulation', *Hormones*. Hormones, 17(4), pp. 507–520. doi: 10.1007/s42000-018-0065-x.
2) Al-sharefi, A. (2016) 'Late Onset Diabetes of Adults (LADA) Masked By Co-Existed Adrenal Failure in the Context of Autoimmune Polyglandular Syndrome 2', *Journal of Diabetes, Metabolic Disorders & Control*. doi: 10.15406/jdmdc.2016.03.00070.
3) Aschbacher, K. O'Donovan, A., Wolkowitz, O., Dhabhar, F., Su, Y. & Epel, E. (2013) 'Good stress, bad stress and oxidative stress: Insights from anticipatory cortisol reactivity', *Psychoneuroendocrinology*. doi: 10.1016/j.psyneuen.2013.02.004.
4) Barnes, P. J. (2015) 'Anti-inflammatory Actions of Glucocorticoids: Molecular Mechanisms', *Clinical Science*. doi: 10.1042/cs0940557.
5) Bernatova, I., Puzserova, A., Balis, P., Sestakovsa, N., Korvathova, M., Kralovicova, Z. and Zitnanova, I. (2018) 'Chronic stress produces persistent increases in plasma corticosterone, reductions in brain and cardiac nitric oxide production, and delayed alterations in endothelial function in young prehypertensive rats', *Frontiers in Physiology*. doi: 10.3389/fphys.2018.01179.
6) Van Boxelaere, M., Clements, J., Callaerts, P., D'Hooge, R. and Callaerts-Vegh, Z. (2017) 'Unpredictable chronic mild stress differentially impairs social and contextual discrimination learning in two inbred mouse strains', *PLoS ONE*. doi: 10.1371/journal.pone.0188537.
7) Carney, R. M. and Freedland, K. E. (2017) 'Depression and coronary heart disease', *Nature Reviews Cardiology*. doi: 10.1038/nrcardio.2016.181.
8) Chatterjee, S. (2016) 'Oxidative Stress, Inflammation, and Disease', in *Oxidative Stress and Biomaterials*. doi: 10.1016/B978-0-12-803269-5.00002-4.

9) Clark, A. R. (2007) 'Anti-inflammatory functions of glucocorticoid-induced genes', *Molecular and Cellular Endocrinology*. doi: 10.1016/j.mce.2007.04.013.

10) Cohen, S., Janicki-Deverts, D., Doyle, W. J., Miller, G. E., Frank, E., Rabin, B. S. and Turner, R. B. (2012) 'Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk.', *Proceedings of the National Academy of Sciences of the United States of America*, 109(16), pp. 5995–9. doi: 10.1073/pnas.1118355109.

11) Cruz-Topete, D. and Cidlowski, J. A. (2014) 'One hormone, two actions: Anti- And pro-inflammatory effects of glucocorticoids', *NeuroImmunoModulation*. doi: 10.1159/000362724.

12) Duric, V., Clayton, S., Leong, M. L. and Yuan, L. L. (2016) 'Comorbidity Factors and Brain Mechanisms Linking Chronic Stress and Systemic Illness', *Neural Plasticity*. doi: 10.1155/2016/5460732.

13) Eisenmann, E. D., Rorabaugh, B. R. and Zoladz, P. R. (2016) 'Acute stress decreases but chronic stress increases myocardial sensitivity to ischemic injury in rodents', *Frontiers in Psychiatry*, 7(APR). doi: 10.3389/fpsyt.2016.00071.

14) Eltzschig, H. K. and Eckle, T. (2011) 'Ischemia and reperfusion-from mechanism to translation', *Nature Medicine*. doi: 10.1038/nm.2507.

15) Frisbee, J. C., Brooks, S. D., Stanley, S. C. and d'Audiffret, A. C. (2015) 'An Unpredictable Chronic Mild Stress Protocol for Instigating Depressive Symptoms , Behavioral Changes and Negative Health Outcomes in Rodents', (December), pp. 1–8. doi: 10.3791/53109.

16) Gavrilović, L., Stojiljkovic, V., Popvic, N., Pejic, S., Todorovic, A., Pavlovic, I. and Pajovic, S. B. (2018) 'Animal Models for Chronic Stress-Induced Oxidative Stress in the Spleen: The Role of Exercise and Catecholaminergic System', in *Experimental Animal Models of Human Diseases - An Effective Therapeutic Strategy*. doi: 10.5772/intechopen.70008.

17) Golbidi, S., Frisbee, J. C. and Laher, I. (2015) 'Chronic stress impacts the cardiovascular system: animal models and clinical outcomes', *American Journal of Physiology-Heart and*

Circulatory Physiology. doi: 10.1152/ajpheart.00859.2014.

18) Goldstein, D. S. and Kopin, I. J. (2007) 'Evolution of concepts of stress', *Stress*. doi: 10.1080/10253890701288935.

19) González-Montero, J., Brito, R., Gajardo, A. I. and Rodrigo, R. (2018) 'Myocardial reperfusion injury and oxidative stress: Therapeutic opportunities', *World Journal of Cardiology*. doi: 10.4330/wjc.v10.i9.74.

20) Hannibal, K. E. and Bishop, M. D. (2014) 'Chronic Stress, Cortisol Dysfunction, and Pain: A Psychoneuroendocrine Rationale for Stress Management in Pain Rehabilitation', *Physical Therapy*. doi: 10.2522/ptj.20130597.

21) Hausenloy, D. J., Botker, H., Condorelli, G., Ferdinandy, P., Garcia-Dorado, D., Heusch, G., Lecour, S., van Laake, L. W., Madonna, R., Ruiz-Meana, M., Schulz, R., Sluijter, J. P., Yellon, D. M. and Ovize, M. (2013) 'Translating cardioprotection for patient benefit: Position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology', *Cardiovascular Research*. doi: 10.1093/cvr/cvt004.

22) Headrick, J. P., Peart, J. N., Budiono, B. P., Shum, D. H. K., Neumann, D. L. and Stapelberg, N. J. C. (2017) 'The heartbreak of depression: "Psycho-cardiac" coupling in myocardial infarction', *Journal of Molecular and Cellular Cardiology*. doi: 10.1016/j.yjmcc.2017.03.007.

23) Hoffman, J. W., Gilbert, T. B., Poston, R. S. and Silldorff, E. P. (2004) 'Myocardial reperfusion injury: etiology, mechanisms, and therapies.', *The Journal of extra-corporeal technology*.

24) Jiang, W., Li, Y., Sun, J., Li, L., Li, J. W., Zhang, C., Huang, C., Yang, J., Kong, G. Y. and Li, Z. F. (2017) 'Spleen contributes to restraint stress induced changes in blood leukocytes distribution', *Scientific Reports*. doi: 10.1038/s41598-017-06956-9.

25) Juruena, M. F. (2014) 'Early-life stress and HPA axis trigger recurrent adulthood depression', *Epilepsy and Behavior*. Elsevier Inc., 38, pp. 148–159. doi: 10.1016/j.yebeh.2013.10.020.

26) Kalogeris, T., Baines, C. P., Krenz, M. and Korthuis, R. J. (2014) *Cell Biology of Ischemia/Reperfusion Injury*. doi: 10.1016/B978-0-12-394309-5.00006-7.Cell.

27) Kalra, S., Einarson, A., Karaskov, T., Van Uum, S. and Koren, G. (2007) 'The relationship between stress and hair cortisol in healthy pregnant women', *Clinical and Investigative Medicine*.

28) Khansari, N., Shakiba, Y. and Mahmoudi, M. (2009) 'Chronic Inflammation and Oxidative Stress as a Major Cause of Age- Related Diseases and Cancer', pp. 73–80.

29) de Kloet, E. R., Otte, C., Kumsta, R., Kok, L., Hillegers, M. H., Hasselmann, H., Kliegel, D. and Joels, M. (2016) 'Stress and Depression: a Crucial Role of the Mineralocorticoid Receptor', *Journal of Neuroendocrinology*. doi: 10.1111/jne.12379.

30) Kolassa, I. T., Eckart, C., Ruf, M., Neuner, F., de Quervain, D. J. and Elbert, T. (2007) 'Lack of cortisol response in patients with posttraumatic stress disorder (PTSD) undergoing a diagnostic interview', *BMC Psychiatry*. doi: 10.1186/1471-244X-7-54.

31) Kyrou, I. and Tsigos, C. (2007) 'Stress mechanisms and metabolic complications', in *Hormone and Metabolic Research*. doi: 10.1055/s-2007-981462.

32) Lichtman, J. H., Bigger, J. T., Blumenthal, J. A., Frasure-Smith, N., Kaufmann, P. G., Lesperance, F., Mark, D. B., Sheps, D. S., Taylor, C. B. and Froelicher, E. S. (2009) 'Depression and Coronary Heart Disease: Recommendations for Screening, Referral, and Treatment', *FOCUS*. doi: 10.1176/foc.7.3.foc406.

33) Lochner, A., Genade, S. and Moolman, M. A. (2003) 'Ischemic preconditioning : Infarct size is a more reliable endpoint than functional recovery', 346, pp. 337–346. doi: 10.1007/s00395-003-0427-6.

34) Lu, L., Liu, M., Sun, M., Zheng, Y. and Zhang, P. (2015) 'Myocardial Infarction: Symptoms and Treatments', *Cell Biochemistry and Biophysics*. doi: 10.1007/s12013-015-0553-4.

35) Lu, X. T., Liu, Y. F., Zhang, L., Yang, R. X., Liu, X. Q., Yan, F. F., Wang, Y. B., Bai, W. W., Zhao, Y. X. and Jiang, F. (2012) 'Unpredictable chronic mild stress promotes atherosclerosis in high cholesterol-fed rabbits', *Psychosomatic Medicine*. doi: 10.1097/PSY.0b013e31825d0b71.

36) Mitchell, J. (1973) 'Lymphocyte Circulation in the Spleen: Marginal zone bridging channels

and their possible role in cell traffic', pp. 93–107.

37) Nicolaides, N. C., Kyratza, E., Lamprokostopoulou, A., Chrousos, G. P. and Charmandari, E. (2015) 'Stress, the Stress System and the Role of Glucocorticoids', *Neuroimmunomodulation*, 22(1–2), pp. 6–19. doi: 10.1159/000362736.

38) Padgett, D. A. and Glaser, R. (2003) 'How stress influences the immune response', *Trends in Immunology*. doi: 10.1016/S1471-4906(03)00173-X.

39) Sauvé, B., Koren, G., Walsh, G., Tokmakejian, S. and Van Uum, S. H. (2007) 'Measurement of cortisol in human hair as a biomarker of systemic exposure', *Clinical and Investigative Medicine*.

40) Sheikh, A. S., Yahya, S., Sheikh, N. S. and Sheikh, A. A. (2012) 'C-reactive protein as a predictor of adverse outcome in patients with acute coronary syndrome', *Heart Views*. doi: 10.4103/1995-705x.96660.

41) Sorrells, S. F. and Sapolsky, R. M. (2007) 'An inflammatory review of glucocorticoid actions in the CNS', *Brain, Behavior, and Immunity*. doi: 10.1016/j.bbi.2006.11.006.

42) STRATAKIS, C. A. and CHROUSOS, G. P. (1995) 'Neuroendocrinology and Pathophysiology of the Stress System', *Annals of the New York Academy of Sciences*. doi: 10.1111/j.1749-6632.1995.tb44666.x.

43) Straub, R. H. and Cutolo, M. (2016) 'Glucocorticoids and chronic inflammation', *Rheumatology (United Kingdom)*, 55, pp. 116–1114. doi: 10.1093/rheumatology/kew348.

44) Swirski, F. K., Nahrendorf, M., Etzrodt, M., Wildgruber, M., Cortez-Teamozo, V., Panizzi, P., Figueiredo, J. L., Kohler, R. H., Chudnovskiy, A., Waterman, P., Aikawa, E., Mempel, T. R., Libby, P., Weissleder, R. and Pittet, M. J. (2009) 'Identification of splenic reservoir monocytes and their deployment to inflammatory sites', *Science*. doi: 10.1126/science.1175202.

45) Tsigos, C., Kyrou, I., Kassi, E. and Chrousos, G. P. (2000) 'Stress, Endocrine Physiology and Pathophysiology', *Endotext*.

46) Vinten-Johansen, J. (2004) 'Involvement of neutrophils in the pathogenesis of lethal

myocardial reperfusion injury', *Cardiovascular Research*. doi: 10.1016/j.cardiores.2003.10.011.

47) Wang, Y., Liu, X., Zhang, D., Chen, J., Liu, S. and Berk, M. (2013) 'The effects of apoptosis vulnerability markers on the myocardium in depression after myocardial infarction', *BMC Medicine*. doi: 10.1186/1741-7015-11-32.

48) WHO (2016) *Cardiovascular diseases (CVDs) fact sheets*, *Who*. Available at: https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds).

49) Willner, P. (2017) 'The chronic mild stress (CMS) model of depression: History, evaluation and usage', *Neurobiology of Stress*. doi: 10.1016/j.ynstr.2016.08.002.

50) Yang, N., Ray, D. W. and Matthews, L. C. (2012) 'Current concepts in glucocorticoid resistance', *Steroids*. doi: 10.1016/j.steroids.2012.05.007.

51) Yehuda, R. and Seckl, J. (2011) 'Minireview: Stress-related psychiatric disorders with low cortisol levels: A metabolic hypothesis', *Endocrinology*. doi: 10.1210/en.2011-1218.

52) Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., Budaj, A., Pais, P., Varigos, J. and Lisheng, L. (2004) 'Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case control study', *The Lancet*, 364(9438), pp. 937–952. doi: 10.1016/S0140-6736(04)17018-9.

53) Zhu, S., Shi, R., Wang, J., Wang, J. F. and Li, X. M. (2014) 'Unpredictable chronic mild stress not chronic restraint stress induces depressive behaviours in mice', *NeuroReport*. doi: 10.1097/WNR.0000000000000243.

54) Zuidema, M. Y. (2010) 'Ischemia/reperfusion injury: The role of immune cells', *World Journal of Cardiology*. doi: 10.4330/wjc.v2.i10.325.

Appendices

Appendix A

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Approved with Stipulations

Date: 20 June 2018

PI Name: Mr Lucien Sher

Protocol #: 6311

Title: Investigating the effects of chronic stress on cardiovascular function

Dear Lucien Sher

The Investigating the effects of chronic stress on cardiovascular function submission was reviewed on 20 June 2018 by Research Ethics Committee: Animal Care and Use via committee review procedures and was approved on condition that the following stipulations are adhered to:

1. In the response to modifications, the applicant states that the aim of experiment 1 is to "establish the stress model" in their department and "to assess to what extent the rats are actually stressed". Considering this statement, the committee is of the believe that the planning of experiments 3 and 4 (which should now be 2 and 3) is premature. These experiments are dependent on a working stress model.
For this reason, only experiment 1 is approved at this time. Once this stress model is confirmed, the applicant must inform the committee (via submitting a progress report) so that experiments 2 & 3 can be reassessed.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number 6311 on any documents or correspondence with the REC: ACU concerning your research protocol.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

If you have any questions or need further help, please contact the REC: ACU secretariat at wabeukes@sun.ac.za or 021 808 9003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

Appendix B

The following appendix includes the protocol followed for the following ELISAs: **ACTH** (E-EL-R0048), **corticosterone** (E-EL-R0269) and **hs-CRP** (E-EL-R0506). ACTH and corticosterone kits were competitive ELISAs, while hs-CRP was a sandwich ELISA.

Reagent preparation:

- **Standard working solution:** After the standard was centrifuged at 1000 g for 60 seconds (Boeco M240, Hamburg, Germany), 1 mL of plasma sample and standard diluent was added to achieve the appropriate concentration for each kit. Seven additionally serial dilutions were made for the standard curve.

- **Biotinylated Detection Antibody (Ab) working solution:** With the amount of desired wells in mind, a 1x solution was prepared with biotinylated detection Ab diluent (50 µL/well).

- **Horse radish peroxidase (HRP) conjugate working solution:** With the amount of desired wells in mind, a 1x solution was prepared with HRP-conjugate diluent (100 µL/well).

- **Wash buffer:** Add 30 mL of concentrated wash buffer to 720 mL of distilled water (dH_2O).

Assay Procedure (Competitive ELISA): ACTH (E-EL-R0048) and **corticosterone** (E-EL-R0269).

It is important to note that for the competitive ELISA the 96-well plate supplied is coated with the antigen (corticosterone or ACTH).

1. 50 µL of standards and sample were added in duplicate in a 96-well plate. Immediately after, 50 µL of **Biotinylated Detection Ab working solution** was added and the plate was incubated at 37°C for 45 minutes. As a result, the anti-bodies within the sample can compete with the biotinylated detection anti-bodies in binding to the antigen.
2. 350 mL of **wash buffer** was added to wells and allowed to soak for 1 minute. This was repeated three times in total. This is to remove any unbound anti-bodies in the wells.
3. 100 µL of **HRP conjugate working solution** was added to each well and the plate incubated at 37°C for another 30 minutes. HRP is the enzyme that binds to the biotinylated detection Ab.
4. The plate was then washed 5 times as in step 2. This removes any unbound HRP.
5. 90 µL of **substrate reagent** was added to each well, after which the plate was incubated at 37°C for 15 minutes. The reaction between the HRP conjugate and the substrate reagent produce a clear blue color. Less blue color indicates more antibody sample bound to antigens, while the converse holds true.
6. 50 µL of **stop solution** was added to each well immediately after and the plate was read at 450 nm using the micro-plate reader (EZ Read 400 Microplate reader, Biochrom, Holliston MA).

Assay Procedure (Sandwich ELISA): hs-CRP (E-EL-R0506).

It is important to note that for the sandwich ELISA, the 96-well plate supplied is coated with an anti-body designed to bind the antigen under investigation (CRP).

1. 100 µL of standards and sample were added in duplicate in a 96-well plate and the plate was incubated at 37°C for 90 minutes. This allows the antigen of under investigation to bind to the anti-body coated wells.

2. Liquid was removed and 100 µL **biotinylated detection Ab working solution** was added to each well, after which the plate was incubated at 37°C for 60 minutes. The biotinylated detection anti-body then binds to the antigen (creating an anti-body || antigen || anti-body "sandwich").
3. 350 mL of **wash buffer** was added to wells and allowed to soak for 1 minute. This was repeated three times in total. This removes any unbound anti-bodies and antigens.
4. 100 µL of **HRP conjugate working solution** was added to each well and the plate was incubated at 37°C for another 30 minutes. The HRP conjugate bind to the biotinylated detection anti-body.
5. The plate was then washed 5 times as in step 3.
6. 90 µL of **substrate reagent** was added to each well, after which the plate was incubated at 37°C for 15 minutes. The reaction between the HRP conjugate and the substrate reagent produce a clear blue color. Unlike the competitive ELISA, the darker blue color indicates the presence of higher antigen levels in your sample.
7. 50 µL of **stop solution** was added to each well immediately after and the plate was read at 450 nm using the micro-plate reader (EZ Read 400 Microplate reader, Biochrom, Holliston MA).

Appendix C

The following appendix includes protocols for **TBARS** and **SOD** activity.

TBARS: This assay measures natural bi-products of lipid peroxidation, like malondialdehyde (MDA) (Hodges *et al.*, 1999). MDA forms a 1:2 adduct with thiobarbituric acid (TBA) which can be measured colorimetrically.

Reagent preparation:

- **1x Phosphate buffer saline (PBS):**
  - 800 mL of dH_2O + 8 g of NaCl + 0.2 g of KCl + 1.44 g of Na_2HPO_4 + 0.24 g of KH_2PO_4.
  - Adjust pH to 7.4 using HCl.
  - Top up to one liter using dH_2O.
- **Ortho-phosphoric acid (**0.2 M**):**
  - 684 µL orthophosphoric acid (14.62 M) + 40.5 mL H_2O.
- **Butylated hydroxytoluene (BHT) working solution**
  - 8.82 mg BHT + 10 mL EtOH.
- **Tributylamine (TBA)**
  - 0.159 g TBA + 10 mL NaOH.

Tissue sample preparation: (The sample preparation applies for both the TBARS and SOD assays)

1. 100 mg of spleen tissue was added to an Eppendorf tube with 1 mL of **PBS** and homogenized thoroughly**.**
2. The homogenate was sonicated (Misonixa ultrasonic liquid processor S-4000, Hielscher, Germany) for 10 seconds.

3. The sample was centrifuged (Boeco M240, Hamburg, Germany) at 10000 rpm for one minute.

Assay procedure:

1. 50 µL of the supernatant, 12.5 µL **BHT** and 100 µL **ortho-phosphoric acid** were added into a 2 mL microfuge tube.
2. Samples were vortexed for 5 seconds.
3. 12.5 µL **TBA** was added to the microfuge tube.
4. Samples were vortexed for 5 seconds.
5. Samples were placed in a water bath at 90°C for 45 minutes (made sure there was a hole in the lid of the microfuge tube).
6. Samples were allowed to cool down on ice.
7. 1 mL butanol and 100 µL saturated salt solution were added to the microfuge tube.
8. Samples were vortexed for 5 seconds.
9. The samples were centrifuged (Boeco M240, Hamburg, Germany) at 10000 rpm for one minute.
10. The top pink butanol layer was transferred to a 96-well plate (250 µL per well).
11. The plate was read at 532 nm in the Multiskan® Spectrum microplate spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

SOD: This assay measures the auto-oxidation of 6-hydroxydopamine (6-HD) at 490 nm for 5 minutes (with readings every 30 seconds).

Reagent preparation:

- **SOD assay buffer:** 50 mM NaPO4- without Triton X-100, pH 7.4.

- **6-HD:** nitrogen purge 10 mL MilliQ water with 50 µL perchloric acid for 15 minutes. Use 10 mL of this and add 4 mg 6-HD to this solution. Wrap in foil and store on ice.
- **Diethylenetriaminepentaacetic acid (DETAPAC):** 0.4 mg in 10 mL of SOD assay buffer**.**

Assay procedure:

1. 12 µL of sample and blank (dH20) were added in triplicate to the 96-well plate.
2. 15 µL of **6-HD** was added to each well.
3. 170 µL of **DETAPAC** was added to each well to initiate the reaction.
4. Absorbance was measured for 5 minutes at 490 nm using a plate reader (EZ Read 400 Microplate reader, Biochrom, Holliston MA). Readings were recorded every 30 seconds.

Appendix D

Ex vivo heart perfusions

Reagent preparation:

- The following five individual buffers were made and kept at 4°C.

Solution	Buffer	Amount required
1	NaCl (119 mM)	279.00 g/ 2 L
2	$NaHCO_3$ (25 mM)	83.60 g/ 2 L
3	KCl (4.75 mM) $KHPO_4$ (1.2 mM)	17.60 g/ 1 L 8.10 g/ 1 L } **1 L total**
4	$MgSO_4.7H_2O$ (0.6 mM) Na_2SO_4 (0.6 mM)	7.40 g/ 1 L 4.20 g/ 1 L } **1 L total**
5	$CaCl_2.H_2O$ (1.25 mM)	18.00 g/ 1 L

- **Krebs Henseleit bicarbonate buffer:**

1. 250 mL of **solution 1** + 250 of s**olution** 2 + 100 mL of **solution 3** + 100 mL of **solution 4**
2. 9 g of glucose was added.
3. After glucose had dissolved, 50 mL of **solution 5** was added.
4. Krebs buffer was gassed with 5% CO_2 and 95% O_2 to maintain optimal pH (7.4) and oxygenation level.

Animal dissection + heart perfusion

1. The heart was excised and placed in ice-cold Krebs buffer.

2. The aortic arch was cut off to make the canula fit properly.
3. The aorta was placed around top canula and retrogradely perfused (Langendorff) for 15 minutes. It was secured tightly with thin elastic string.
4. During the first 15 minutes, excess fat was trimmed off and a side canula was inserted into pulmonary vein.
5. Subsequently, hearts were switched to the working mode for another 15 minutes. During this time functional parameters were assessed.
6. Before ischemia was induced, the heart was put back into Langendorff mode.
7. The left anterior descending coronary artery was thereafter tied off for 35 minutes using silk suture. During this period hearts were kept at 36.8 °C.
8. To confirm whether the tie was successful, coronary flow should be reduced by approximately 50%.
9. After the 35 minutes, the tie was undone and the heart was allowed to perfuse retrogradely for 10 minutes.
10. Subsequently, hearts were switched to the working mode for 20 minutes to assess the same functional parameters.
11. The heart was thereafter switched back to Langendorff for the final 30 minutes of the protocol.

Staining

Reagent preparation

- **1% triphenyltetrazolium chloride** (TTC):
    - Solution I: 100 mM $NaH_2PO_42H_2O$ (15.6 g / 1 liter dH_20)
    - Solution II: 100 mM Na_2HPO_4 (14.2 g / 1 liter dH_20)
    - 2, 3, 5 – triphenyltertazoliumchloride (TTC)

1. 20 mL of Solution I + 80 mL of Solution II
2. pH to 7.4
3. 5 mL of the above mentioned solution (I + II) and 0.05 g of Tetrazolium salt was used per heart (cover in foil as it is light sensitive).

- **10% formaldehyde**

Staining and drawing protocol

1. Suture was re-tied at the original location.
2. The plastic tube was removed from the top cannula and 0.5 % Evans blue solution was slowly injected (careful to not overstain).
3. The heart was cut from the cannula and frozen overnight at -20°C.
4. The heart was then cut from the apex towards the knot (transverse) in five slices.
5. The five slices of each heart were placed in a plastic tube with the 5 mL of the TTC solution.
6. Samples were heated for 7 minutes using hands (until infarct and area at risk are easily distinguishable).
7. The TTC was poured off and the formaldehyde solution was added for an hour.
8. The slices of a sample were then arranged from big to small between two Perspex slides.
9. The viable tissue (V), area at risk (AR) and infarct zone (I) was sketched onto transparent paper.
10. The paper was scanned and the image was saved as a ‘’.png’’ file.

Calculations using ImageJ

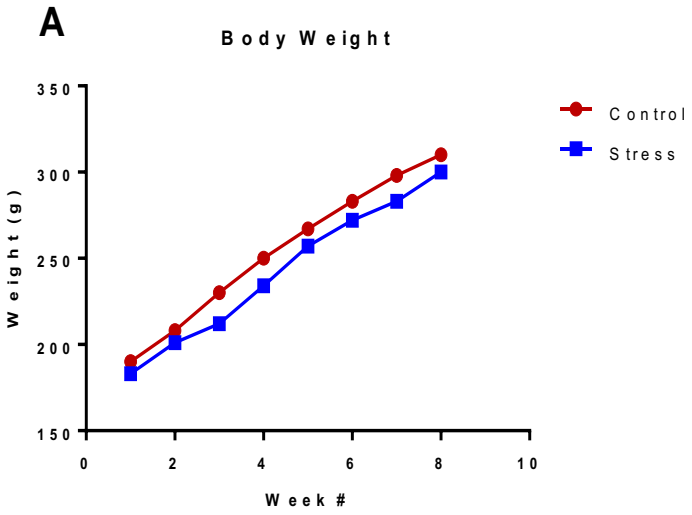
1. The scale was set accordingly: “Distance in pixels”, “Known distance” and “Pixel aspect ratio” was kept constant throughout the analyses. This doesn’t matter too much, as the result is a ratio/percentage, rather than an actual measurement.
2. Thereafter the free hand tool was used to carefully draw around each of the zones of interest.
3. The area was calculated for the respective zones. As volume could not be assessed, heart was sliced identically in all hearts. And the average of all slices calculated.
4. The infarct size was calculated as follows:

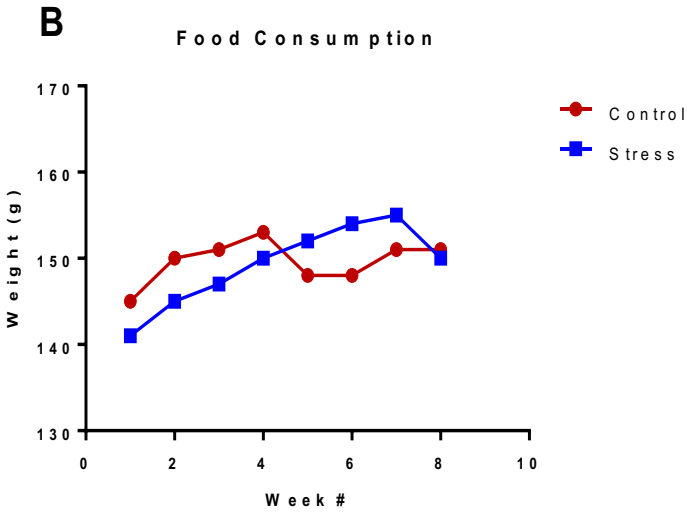
$$Infarct\ size\ \% = \frac{I}{(AR + I)} \times 100$$

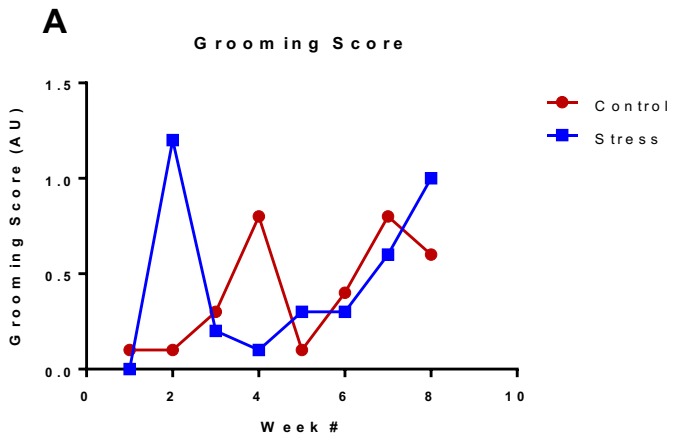
5. The area at risk was calculated as follows:

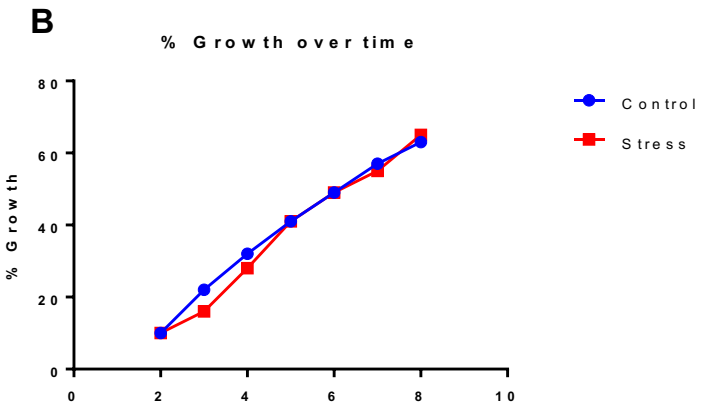
$$Area\ at\ risk\ \% = \frac{(AR + I)}{Total\ area} \times 100$$

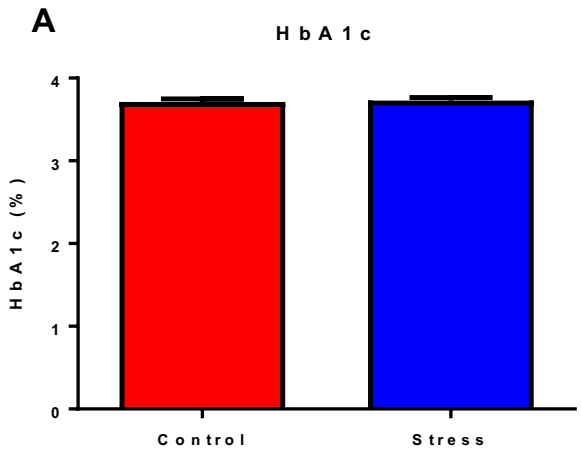
Appendix E

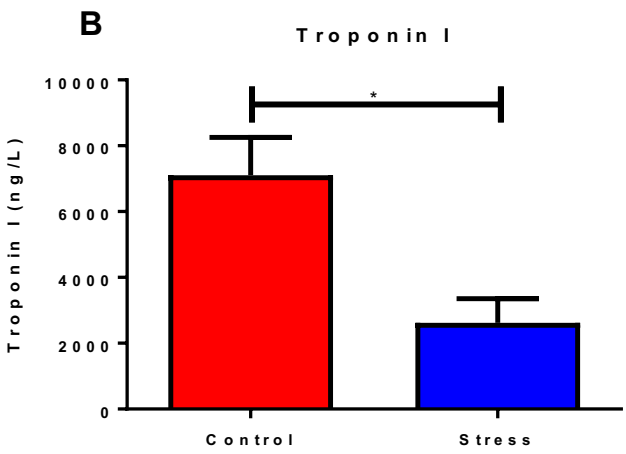


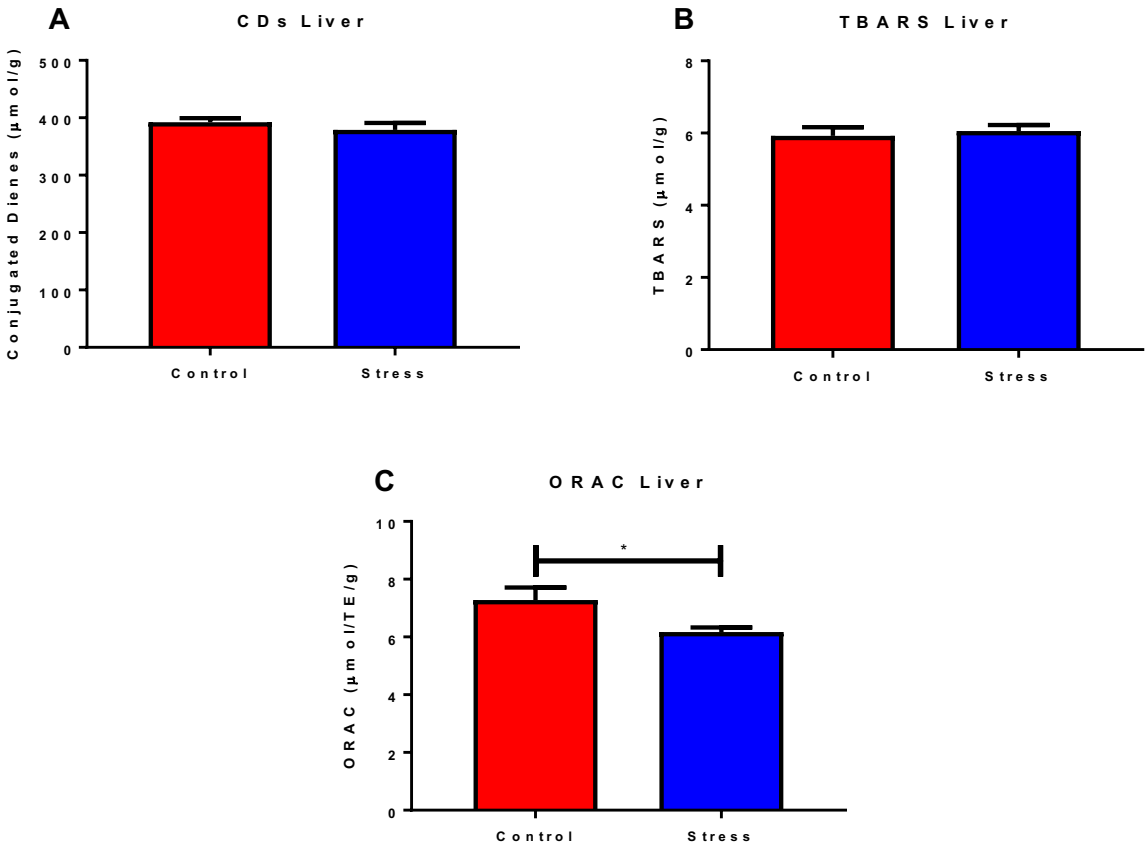


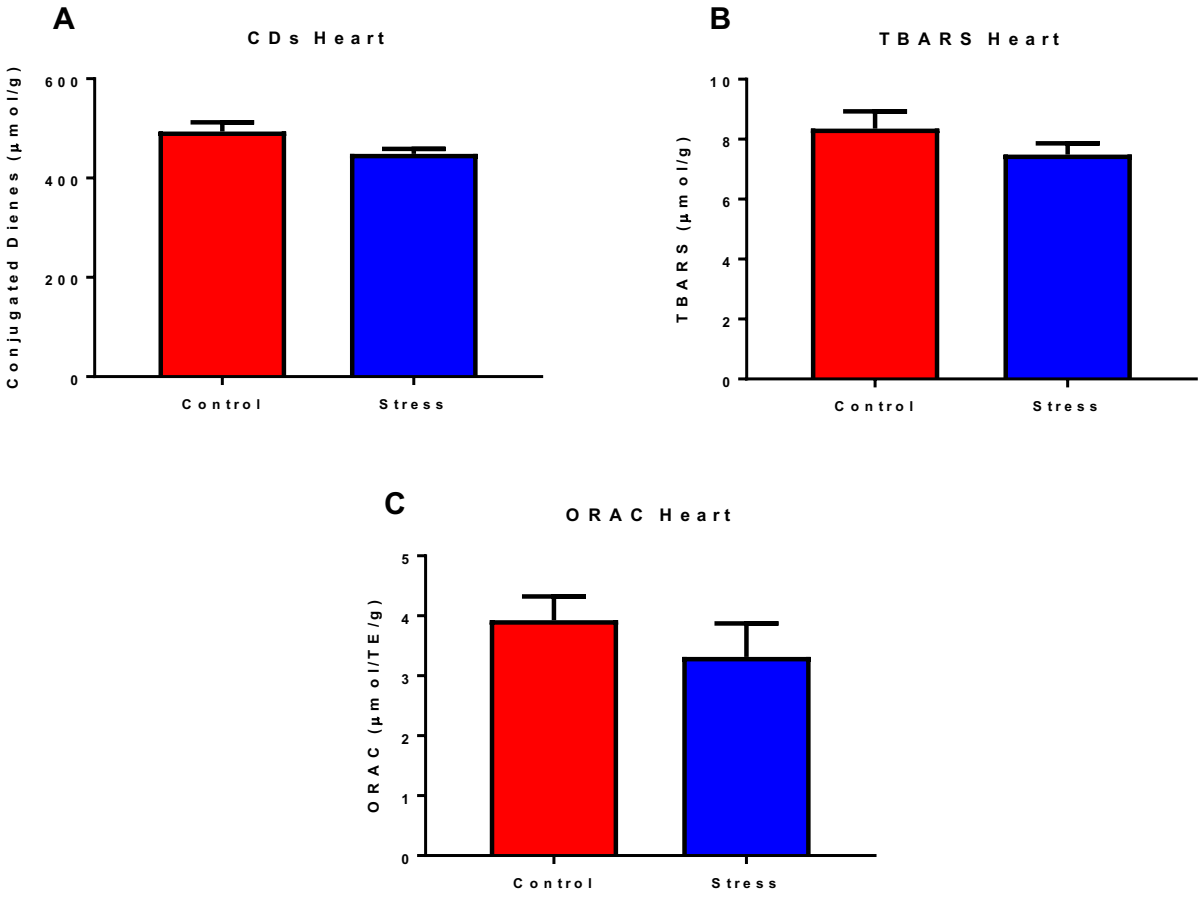


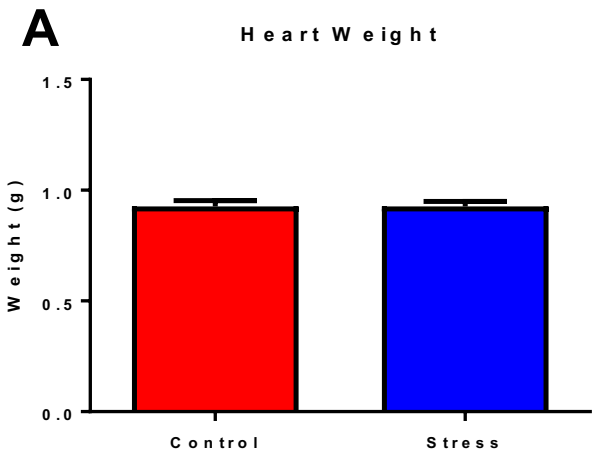


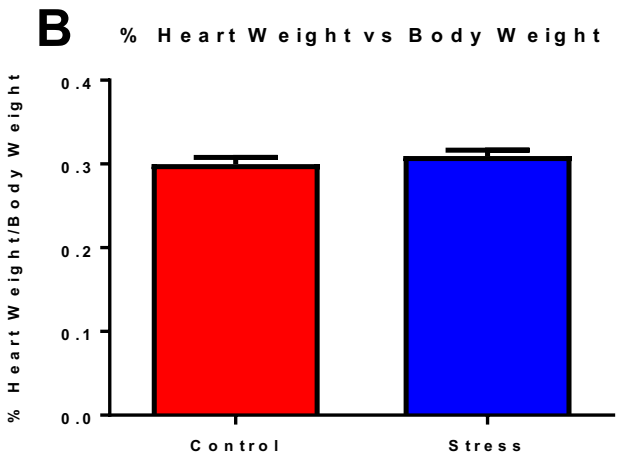


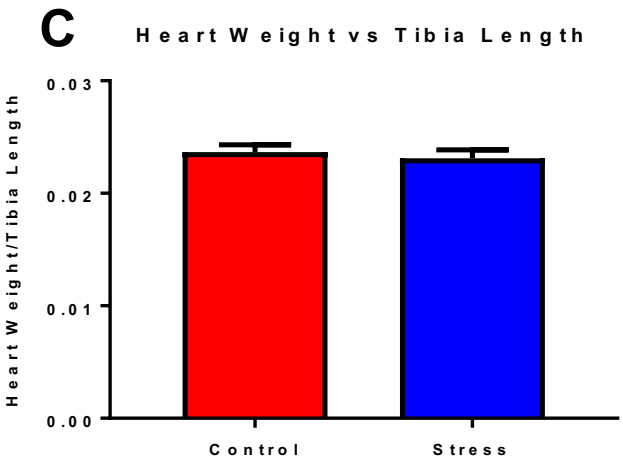


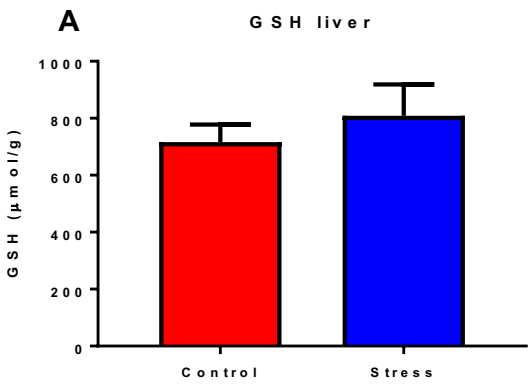


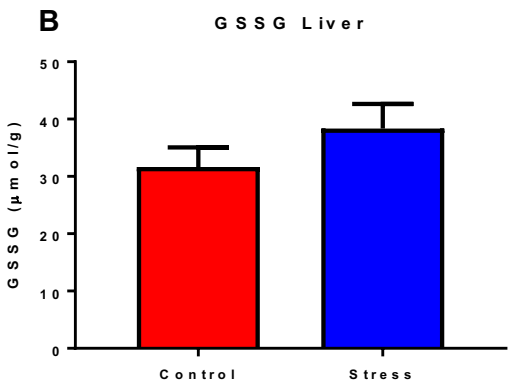


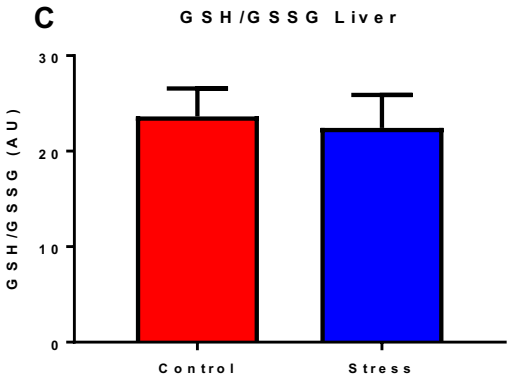


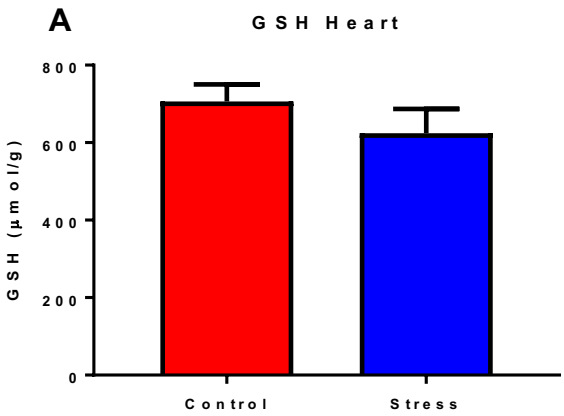


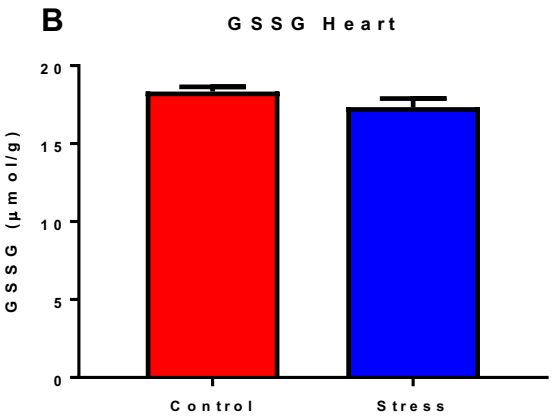


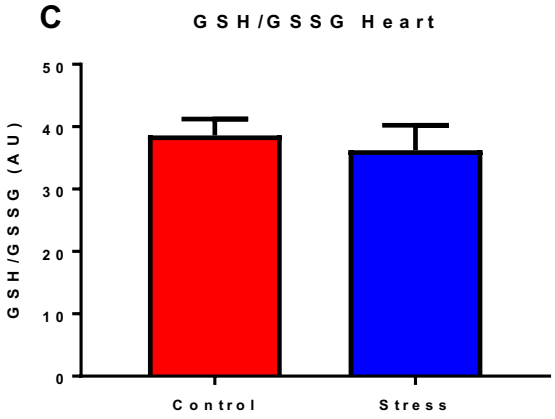












Appendix F